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THE Na^+,K^+-PUMP
Part A: Molecular Aspects

THE Na^+,K^+-PUMP
Part A: Molecular Aspects

Proceedings of the 5th International Conference on Na^+,K^+-ATPase, Held at Fuglsø Conference Center, Denmark, June 14–19, 1987

Editors

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Two-Sided Functional Na^+,K^+-ATPase-Liposomes for Characterizing the Permeability and Side of Action of Pump Inhibitors / *H.G. Rey, P. Meda, and B.M. Anner*

Effect of Primaquine on the Topology of Na^+,K^+-ATPase and the Receptor for Asialoglycoproteins / *G.J. Strous, P. van Kerkhof, A. van Bokhoven, A.L. Schwartz, and J.J.H.H.M. de Pont*

Non-Esterified Fatty Acids and the Circulating Inhibitor of Na^+,K^+-ATPase / *H.G.P. Swarts, J.A.H. Timmermans, F.M.A.H. Schuurmans Stekhoven, J.J.H.H.M. de Pont, S.J. Graafsma, and T.A. Thien*

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Inotropic and Toxic Actions of Several Cardiac Steroids in Sheep Cardiac Tissues / *J. Andrew Wasserstrom and David E. Farkas*

Effect of Apamin on the Na^+,K^+-Pump and Na^+,K^+-ATPase / *Hana Zemková, Jan Teisinger, and Frantisek Vyskočil*

Preface

The idea of a sodium pump in the cell membrane was introduced by R.B. Dean in 1941 in a paper titled, "Theories of electrolyte equilibrium in muscle" (Biol. Symp. 3:331–348). Referring to experiments by L.A. Heppel (1938), by C.L.A. Schmidt (1939), and by H.B. Steinbach (1940), Dean concluded "that the muscle can actively move potassium and sodium against concentration gradients . . . [but] this requires work. Therefore there must be some sort of a pump possibly located in the fiber membrane, which can pump out the sodium or, which is equivalent, pump in the potassium."

In the nearly half century that has passed since Dean's proposal, extensive work has been done by scientists all over the world to understand how a biological system can convert chemical energy from the hydrolysis of adenosine triphosphate (ATP) into a movement of cations against electrochemical gradients. For thirty years it has been known that the Na^+,K^+-pump is identical with membrane-bound Na^+,K^+-ATPase, and a large amount of information has been collected about the system. The increase in knowledge has revealed the complexity of the system, and thus far it has not been possible to determine the nature of molecular events that lead to coupling between the chemical reaction and the translocation reaction.

To facilitate the exchange of information, five international conferences have been held on the subject, the first in 1973 in New York, sponsored by the New York Academy of Sciences; the second in 1978 in connection with Aarhus University, Denmark. Since then it has been a tradition to hold a conference every third year: in 1981 at Yale University, New Haven, CT; in 1984 in Cambridge, England; and in 1987 again in connection with Aarhus University, Denmark.

The 1987 meeting was held June 14–19 at Fugslø Conference Center in Denmark. The conference was organized by F. Cornelius, M. Esmann, I. Klodos, J.G. Nørby, J.C. Skou (Institute of Biophysics), and A.B. Maunsbach (Department of Cell Biology, Institute of Anatomy), University of Aarhus, Denmark. On behalf of the organizers, we thank all who took care of the practical arrangements, and those who helped to run the conference. We wish to express our special thanks to Edith Moldt and Jytte Kragelund for their excellent secretarial assistance.

This volume, Part A, is devoted to molecular aspects of the Na^+,K^+-pump. It contains the papers presented at the conference that deal with amino acid sequence, structure, and enzymatic and transport mechanisms of Na^+,K^+-ATPase. Part A also contains a section where this system is compared with other transport ATPases.

The accompanying volume, Part B, presents the papers regarding the cellular and physiological aspects of the Na^+,K^+-pump: biosynthesis and molecular biology; the physiological regulation of Na^+,K^+-transport; Na^+,K^+-homeostasis; the role of Na^+,K^+-transport in secondary active transport in specialized tissues like the kidney, skeletal muscle, and heart muscle; and the Na^+,K^+-pump as receptor for cardiac glycosides. Part B also includes a historical survey of the concept of active transport.

The two volumes give an overview of the present status in the field and also of the problems to be solved before it is possible to understand the molecular events underlying the coupling between the chemical reaction and transport.

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Acknowledgments

On behalf of all the participants, the organizing committee of the Fifth International Conference on Na^+,K^+-ATPase, held at Fuglsø June 14–19, acknowledge the generous financial support from: The Danish Ministry of Education, University of Aarhus Research Foundation, The Danish Medical Research Council, The Danish Science Research Council, The Novo Foundation, The Leo Foundation, The M. Møller Foundation, The M.C. Holst Foundation, and the following companies: Provinsbanken A/S, Handelsbanken A/S, Sparekassen SDS, Nordisk Gentofte A/S, Upjohn, Janssenpharma A/S, Packard Instruments, and Ceres Breweries A/S.

Looking for the answer.

You hunt it,
you catch it,
you fool yourself;
the answer,
is always,
a step ahead.

—J.C.S.

Amino Acid Sequence and Structure

The Na^+,K^+-Pump, Part A: Molecular Aspects, pages 3-18
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OVERVIEW: AMINO ACID SEQUENCES OF THE α AND β SUBUNITS OF THE Na,K-ATPase

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INTRODUCTION

Since the discovery of the Na,K-ATPase by Skou in 1957, intense efforts have been directed at determining its structure and the mechanism by which it transports Na and K across the plasma membrane. Determination of the amino acid sequences of the α and β subunits, a necessary prerequisite for a detailed understanding of the higher order structure of the enzyme and its mechanism of action, was attempted by direct sequencing of proteolytic fragments, but these efforts provided only limited portions of the sequence (Collins et al., 1983). However, during the past several years, a number of groups have applied recombinant DNA procedures to this problem. As a result of these studies, there is now a wealth of information concerning the structure of various isoforms of the Na,K-ATPase. In this paper, we review the information presented in these cloning studies and discuss several hypotheses concerning the structural and functional domains of the enzyme.

MOLECULAR CLONING OF MULTIPLE ISOFORMS OF THE Na,K-ATPase

The complete amino acid sequences for the α and β subunits from a number of species have been determined by analysis of their complementary DNAs. The first reports to appear were those concerning the characterization of cDNAs for the α and β subunits of sheep kidney (Shull et al., 1985; Shull et al., 1986a), Torpedo californica electric organ (Kawakami et al., 1985; Noguchi et al.,

1986) and pig kidney (Ovchinnikov et al., 1986). In each of these studies the screening strategy involved the use of oligonucleotide hybridization probes corresponding to various amino acid sequences of the two subunits. An alternative strategy, involving an antibody screening procedure, was used to isolate a cDNA for the rat β-subunit (Mercer et al., 1986) and a partial cDNA for the rat α-subunit (Schneider et al., 1985). cDNAs for the human Na,K-ATPase α (Kawakami et al., 1986a) and β (Kawikami et al., 1986b) subunits were isolated by cross hybridization with α and β cDNAs of the Torpedo californica enzyme.

The initial cloning studies provided a great deal of information about the general structure of the enzyme but did not address the issue of multiple isoforms of the Na,K-ATPase. Based on biochemical evidence it was clear that in rat brain there are at least two isoforms of the catalytic subunit (Sweadner, 1979). These isoforms, termed α and α(+), differ in cardiac glycoside sensitivity (Sweadner, 1979), Na affinity (Lytton, 1985a) and N-terminal amino acid sequence (Lytton, 1985b). In order to determine the amino acid sequences of the rat α and α(+) isoforms we prepared and screened cDNA libraries from rat kidney and brain. In addition to cDNAs encoding the α and α(+) isoforms, we identified a cDNA encoding an isoform, termed αIII, which had not been identified previously (Shull et al., 1986b).

In order to maximize the probability of obtaining full-length cDNAs for the different isoforms of the catalytic subunit, size-fractionated cDNA was used during construction of the libraries. Size analysis of the cDNA was performed following first strand synthesis (Fig. 1, left) and following fractionation of the double stranded cDNA (Fig. 1, right). Three cDNA fractions were isolated from an agarose gel and the middle fractions (3-6 kilobases) were tailed with deoxycytidine 5'-triphosphate and annealed to G-tailed PstI cut pBR322. The brain and kidney libraries, each consisting of 50,000 colonies, were screened using the sheep kidney α-subunit cDNA isolated previously (Shull et al., 1985).

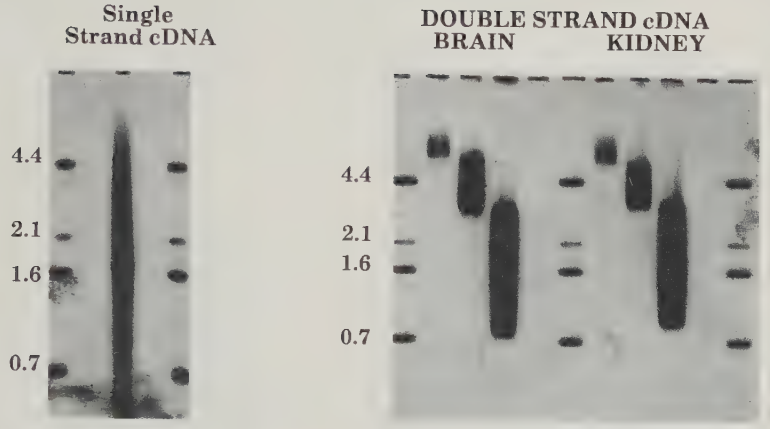

Figure 1. cDNA size analysis. The size distribution of single strand cDNA was analyzed by electrophoresis on an alkaline agarose gel followed by autoradiography (left panel). The second strand synthesis was then performed and the products of that reaction were fractionated on a nondenaturing agarose gel. The right panel shows the size distribution of the three fractions collected from the brain and kidney cDNA. The size of the markers (in kilobase pairs) is indicated.

A total of 117 positive signals were identified in the kidney library and 78 positive signals in the brain library. Eight kidney and 25 brain cDNAs were isolated and analyzed by restriction mapping and blot hybridization analysis. The initial step in this analysis was to examine the cDNAs for restriction fragment length polymorphisms in the regions encoding the phosphorylation site and the ATP binding site. Oligonucleotides corresponding to amino acid sequences at the phosphorylation site (Cys-Ser-Asp-Lys-Thr-Gly-Thr, residues 367-373) and ATP binding site (Thr-Gly-Asp-Gly-Val-Asn, residues 708-713) were used as hybridization probes on Southern blots of α-subunit cDNAs that had been digested with the restriction enzyme HpaII. Figure 2

illustrates the pattern observed when the blots were

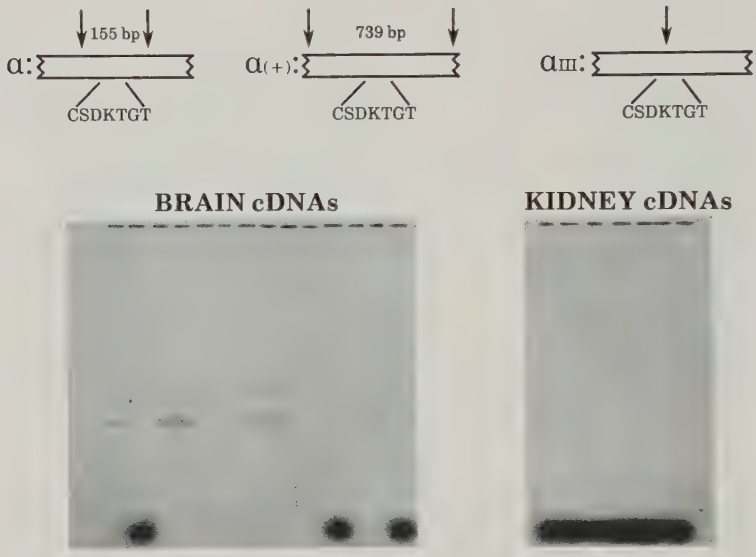

Figure 2. Restriction fragment length polymorphism analysis of brain and kidney α-subunit cDNAs. Brain and kidney cDNAs were digested with the restriction endonuclease HpaII, which has a four base pair recognition sequence. Southern blot hybridization analysis was performed using an oligonucleotide probe derived from the phosphorylation site region (see text). All of the kidney clones and some of the brain clones exhibited a hybridization signal at 155 base pairs. A second group of brain cDNAs had a 739 base pair band and a third group did not hybridize with the probe following digestion with HpaII. The schematic diagram above the autoradiograms illustrates the location of HpaII sites in each of the cDNA classes in relation to the codons for the phosphorylation site region.

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probed with the phosphorylation site oligonucleotide. All of the kidney cDNAs exhibited a single pattern, in which the phosphorylation site was encoded on a 155 base pair HpaII fragment. These cDNAs correspond to the α-isoform.

Three patterns were observed among the brain cDNAs. Six of the 25 brain cDNAs had the same pattern as the kidney cDNAs, sixteen had a 739 base pair fragment which contained the region encoding the phosphorylation site and three cDNAs gave no signal, indicating that the target sequence had been cut by HpaII. These data suggested that there were at least three isoforms of the catalytic subunit.

Detailed restriction maps of cDNAs from each class were determined and their nucleotide sequences were analyzed (see Figures 1-4 of Shull et al., 1986b). The brain and kidney α-isoform cDNAs encode identical primary translation products of 1023 amino acids. Amino acids 6-19 are identical to the first 14 amino acids of the rat brain α-isoform N-terminus determined previously (Lytton, 1985b), indicating that five amino acids are removed from the N-terminus by posttranslational processing. The mature protein consists of 1018 amino acids and has a molecular weight of 112,573. The primary translation product of the α(+) mRNA, deduced from its cDNA, consists of 1020 amino acids. Its identity was confirmed by comparison of the deduced amino acid sequence with that of the N-terminus determined by amino acid sequence analysis of the protein (Lytton, 1985b). Five amino acids are apparently removed from the N-terminus of the α(+) primary translation product to yield a mature protein of 1015 amino acids with a molecular weight of 111,736. The third cDNA had a 1013 codon open reading frame and exhibited high sequence similarity to the α and α(+) isoforms. It seems clear from the amino acid homology that the corresponding mRNA encodes an isoform (αIII) of the catalytic subunit. The molecular weight of the primary translation product is 111,727.

There is very little biochemical data concerning the existence of multiple isoforms of the β-subunit. Differences in molecular weight of the glycosylated β-subunits of rat brain and kidney have been observed but the molecular weights of the protein moieties appear to be the same (Sweadner and Gilkeson, 1985). To determine whether heterogeneity occurs in the amino acid sequences of brain and kidney β-subunits, cDNA libraries were constructed from mRNA of these tissues and screened at low stringency with a sheep kidney β-subunit cDNA (Young et al., 1987). Twenty brain and ten kidney cDNAs were

isolated and extensively characterized by restriction endonuclease mapping. The cDNAs could be grouped into five classes based on the lengths of their 5' and 3' untranslated sequences. Variation in length at the 3' end was due to the use of multiple polyadenylation sites and variation at the 5' end was due to the use of alternative promoters during transcription of the gene. The hybridization patterns observed during Northern blot analysis of mRNA from the two tissues correlated well with the classes of cDNAs isolated. Although these data clearly demonstrated the existence of multiple β-subunit mRNAs, DNA sequence analysis demonstrated that the amino acid sequences encoded by the brain and kidney mRNAs are identical. This, of course, does not rule out the possibility that a different β-subunit isoform is expressed in these tissues. cDNAs for a low abundance isoform could have been missed during screening of the library. Also, if the sequence of a second β-subunit isoform had diverged sufficiently from that of the sheep kidney β subunit, its cDNA might not cross-hybridize with the probe under the conditions utilized.

TRANSMEMBRANE ORGANIZATION OF THE α AND β SUBUNITS

There is a considerable amount of information concerning the location of various residues of the α and β subunits with respect to the membrane. For the α-subunit it is clear that the phosphorylation site and the sites that bind fluorescein isothiocyanate (FITC) (Farley et al., 1984; Kirley et al., 1984) and 5'-[p-(fluorosulfonyl)benzoyl] adenosine (FSBA) (Ohta et al., 1986), which are thought to form part of the ATP binding site, must be located on the cytoplasmic side of the membrane. The position of a number of conformation-dependent proteolysis sites of the α-subunit, which are known to be located on the cytoplasmic side of the membrane, have also been determined (Jørgensen and Collins, 1986). These include Arg 262, Arg 438 and a residue located within the sequence Lys 18-Lys 30. Proteolysis studies of the β-subunit suggest that its N-terminus is located on the cytoplasmic side of the membrane (Geering et al., 1985) and the three potential N-linked glycosylation sites (Asn residues 157, 192 and 264) all appear to be glycosylated and therefore must be on the extracellular side (Shull et al., 1986a; Ovchinnikov et al., 1986). The known location of these sites places

certain constraints on models of the transmembrane organization that are based on analysis of the hydrophobicity of the peptide chains.

The locations of hydrophobic regions which might represent transmembrane domains can be estimated by hydropathy analysis (Kyte and Doolittle, 1982). Hydropathy plots for the β-subunit (Shull et al., 1986a; Noguchi et al., 1986) reveal a single hydrophobic domain (amino acids 34-60) that appears likely to traverse the membrane. Thus, it appears that the β-subunit has a single transmembrane domain, that the N-terminus is located on the cytoplasmic side of the membrane and that the C-terminus is located on the extracellular side. The organization of the α-subunit is more complex and far less certain. Hydropathy analysis of the sheep kidney α-subunit indicates that there may be a total of eight transmembrane domains. Four of these domains occur in the N-terminal third of the protein and their arrangement is consistent with the location of the sites discussed above. The arrangement of the transmembrane domains in the C-terminal regions of the protein is not known. Based on hydropathy analysis of the sheep kidney α-subunit, we suggested that there may be four transmembrane segments between amino acids 780 and 971 (Shull et al., 1985). However, only two transmembrane domains were predicted in this region of the Torpedo α-subunit (Kawakami et al., 1985). Comparisons of the hydropathy plot of the α subunit with those of the Ca-ATPase (MacLennan et al., 1985; Shull et al., 1985) and H,K-ATPase (Shull and Lingrel, 1986) strongly indicate that all three enzymes have the same transmembrane organization. A consideration of the hydropathy data suggests that the C-terminal regions are likely to contain four to six transmembrane domains.

AMINO ACID SIMILARITY COMPARISONS

Amino acid sequence data are now available for three isoforms of the Na,K-ATPase catalytic subunit and for a number of other transport ATPases of the aspartyl phosphate class. These include the sarcoplasmic reticulum Ca-ATPases (MacLennan et al., 1985; Brandl et al., 1986), the H,K-ATPase of the stomach (Shull and Lingrel, 1986), the yeast plasma membrane H-ATPase (Serrano et al., 1986) and the K-ATPase from E.coli (Hesse et al., 1984). Amino

acid sequence comparisons of these proteins can provide insights regarding their structural and functional domains. Figure 3 is a similarity plot comparison of α vs

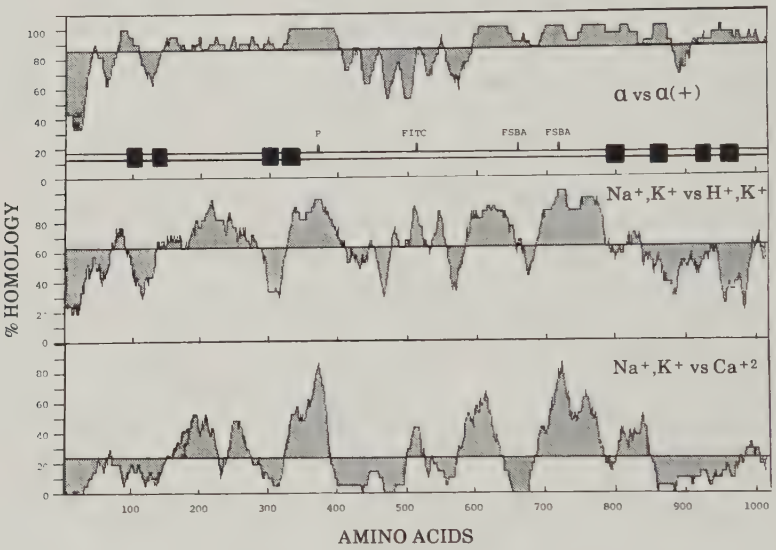

Figure 3. Similarity plot comparisons of the rat Na,K-ATPase α and α(+) isoforms (top panel), sheep kidney Na,K-ATPase α vs rat stomach H,K-ATPase (middle panel) and sheep kidney Na,K-ATPase α vs the slow twitch isoform of the sarcoplasmic reticulum Ca-ATPase (bottom panel). Amino acid alignments were performed and the percent similarity was plotted using a window of 9 amino acids. The data point for each amino acid is the percent identity for residues n-4 to n+4 where n represents the amino acid residue plotted (the program for this analysis was written by Greg Wernke). A linear map of the Na,K-ATPase α-subunit is illustrated in the top panel. The location of probable transmembrane domains (black boxes), the phosphorylation site (P) and regions which bind fluorescein isothiocyanate (FITC) and 5'-[p-(fluorosulfonyl) benzoyl] adenosine (FSBA) are indicated.

α(+), Na,K-ATPase α vs H,K-ATPase and Na,K-ATPase α vs Ca-ATPase. The overall amino acid identity between the three pairs of enzymes is 86%, 62% and 24%, respectively. From these comparisons the regions which have greater or less than the average similarity can be identified. The peak centered around the phosphorylation site clearly reflects the importance of this domain in the catalytic activity of this family of transport ATPases. This domain serves a general function in ion transport rather than an ion-specific function. However, the sequences of the transmembrane domains are highly conserved between isoforms of the Na,K-ATPase but exhibit virtually no similarity between the Na,K-ATPase and Ca-ATPase. It would appear that residues in these domains serve an ion-specific function. The region centered around Asp 710 in the Na,K-ATPase, which is apparently involved in the binding of ATP (Ohta et al., 1986), is highly conserved among ATPases of different ion specificity. The greater than average similarity at the FITC site may also be due to a role in ATP binding (Farley et al., 1984).

There are additional regions having greater than average similarity for which the functions are unknown. A major peak spanning about 50 amino acids is centered around the sequence Val-Thr-Gly-Asp (amino acids 609-612), which is located between the FITC site and the two sites which bind FSBA. This sequence is identical to the sequence 707-710 which is part of one of the FSBA binding peptides and appears to be a critical component of the ATP binding site (Dzhandzhugazyan et al., 1987). A more detailed comparison (Figure 4) suggests that the similarity extends throughout the two domains. Furthermore, the secondary structure for both domains appears to consist of alternating α-helix and β-sheet. An intriguing possibility is that the domain which includes residues 596-641 may be part of the putative second nucleotide binding site. It has been proposed that ATP and fatty acid coenzyme A derivatives may modulate activity of the enzyme by binding at a regulatory site that is distinct from the active site (Askari, 1987; Huang et al., 1986).

Figure 4. Comparison of the amino acid sequences and secondary structures of two domains of the catalytic subunit. The domain that includes residues 695-741 appears to be part of the ATP binding site (see text). The function of the domain that includes residues 596-641 is not known. Identical residues have been boxed; gaps have been included to maximize similarity. The predicted secondary structure is indicated above or below the sequence (X represents α-helix, - represents β-sheet, > represents turns and * represents coil).

A forty amino acid sequence between the fifth and sixth hydrophobic domains (amino acids 807-844) exhibits a high degree of similarity between the Na,K and Ca pumps. However, the functions of domains in the C-terminal regions are unknown. The function of conserved cytoplasmic domains between the second and third transmembrane domains are also unknown, but it has been proposed that this region might be involved in transmission of conformational changes during the catalytic cycle (MacLennan, et al., 1985).

CARDIAC GLYCOSIDE BINDING

Species and isoform differences in cardiac glycoside sensitivity have long been known. The α isoform from sheep and pig and the α(+) isoform from rat are highly sensitive to ouabain, whereas the rat α isoform is resistant. Recent data clearly demonstrate that the α subunit of a resistant species is capable of conferring ouabain resistance to sensitive cells (Kent et al., 1987). Although it is possible that amino acid sequence differences on the cytoplasmic side of the membrane or in the transmembrane domains may be involved, it is more likely that the ouabain insensitivity of the rat α isoform

is due to amino acid substitutions in the extracellular domains. A comparison of the potential extracellular sequences of the sheep α, pig α and the rat α, α(+) and αIII isoforms is illustrated in Figure 5. Of the 24 amino

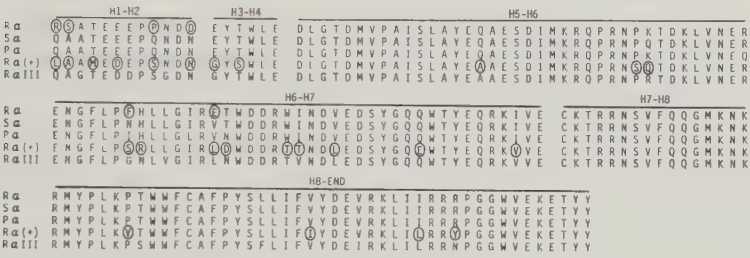

Figure 5. Comparison of the potential extracellular sequences of α subunit isoforms from rat (Rα, Rα(+) and RαIII; data from Shull et al., 1986b), sheep (Sα; data from Shull et al., 1985) and pig (Pα; data from Ovchinnikov et al., 1986). H1-H2 etc. refer to the junctions between the putative transmembrane domains. The circled Rα amino acids indicate those residues in the ouabain resistant rat α sequence which differ from the corresponding residue of the sensitive sheep or pig α isoform. The circled Rα(+) amino acids indicate those residues in the ouabain sensitive Rα(+) sequence which differ from the corresponding residue of the resistant Rα sequence.

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acid differences between rat α and sheep or pig α, only 6 are located in potential extracellular regions. Four of these residues are clustered in the junction between the first and second transmembrane domains (H1-H2). It seems likely that one or more of these four amino acid differences are responsible for the relative insensitivity of the rat α subunit. A comparison of the rat α and α(+) isoforms supports this hypothesis. There are 24 amino acid differences between these isoforms that are located in potential extracellular domains. At most of these positions, the resistant rat enzyme has the same amino acid as the sensitive pig and sheep enzymes. However, in the H1-H2 junction, there are six amino acid differences

between the two isoforms. All of the sensitive enzymes have uncharged amino acids at the boundaries with the transmembrane domains, whereas the rat α isoform has charged amino acids. In addition, rat α has a Pro residue that is not found in the sensitive enzymes.

Although the H1-H2 junction may be involved in ouabain binding, it is clear from previous studies that other regions of the protein are likely to contribute to the ouabain binding site. Studies using photoaffinity derivatives of ouabain suggest the involvement of the H3-H4 junction and sequences in the C-terminal regions (Jørgensen et al., 1982; Goeldner et al., 1983). These data have led to the proposal (Shull et al., 1986b) that the H3-H4 junction might interact with the steroid moiety, that the H1-H2 junction may interact with the lactone ring and that the carbohydrate group may bind to sequences in the C-terminal regions. These hypotheses can be tested using site-directed mutagenesis techniques.

A HYPOTHESIS REGARDING THE CONTROL OF CATION BINDING

Cation binding and occlusion have been heavily studied but little is known about the functional domains that mediate this process. There is some evidence that these domains may be located in the N-terminal regions of the catalytic subunit. An examination of this region of the Na,K-ATPase reveals that it is highly variable in different species and isoforms (Figure 6) yet has some distinctive features. A sequence which begins at Lys 16 is highly charged and is rich in lysine residues. The H,K-ATPase of the gastric mucosa has a similar lysine-rich sequence. It has been shown previously that trypsin cleavage at a site (T2) located near the N-terminus (see Castro and Farley, 1979; Jørgensen et al., 1982; Jørgensen and Collins, 1986) shifts the conformational equilibrium toward the E1 form. The most likely location of this site, which is more accessible in the E1Na conformation than in the E2K form, is in the region including Lys 18 and Lys 30. Cleavage at this site appears to remove a His residue (His 14) which is important in the E1-E2 equilibrium (Skou and Esman, 1980). The variation in accessibility of T2 demonstrates that movement of the lysine-rich sequence occurs during the conformational shifts which accompany cation binding and occlusion. It is possible that the lysine rich domain functions as an

ion-selective gate which controls the passage of cations to and from their binding sites.

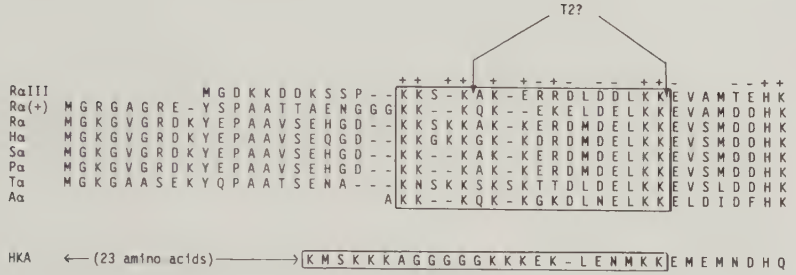

Figure 6. Comparison of the N-terminal amino acid sequences of the Na,K-ATPase α-subunit isoforms from various species. The data shown are for the rat (RαIII, Rα(+), and Rα; data from Shull et al., 1986b), human (Hα; data from Kawakami et al., 1986b), sheep (Sα; data from Shull et al., 1985), pig (Pα; data from Ovchinnikov et al., 1986), Torpedo (Tα; data from Kawakami et al., 1985) and Artemia salina (Aα; Morohashi and Kawamura, 1984). Also shown is a portion of the N-terminal sequence of the rat H,K-ATPase (Shull and Lingrel, 1986). The box encloses a lysine rich sequence which might function in the control of cation binding and occlusion (see text). T2 indicates the possible positions of trypsin site 2, which is readily accessible in the E1Na conformation (Jørgensen et al., 1982).

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OVERVIEW: STRUCTURAL BASIS FOR COUPLING OF E_1-E_2 TRANSITIONS IN αβ-UNITS OF RENAL Na,K-ATPase TO Na,K-TRANSLOCATION

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INTRODUCTION

The α-subunit of Na,K-ATPase is a prototype for cation pump proteins in eukaryotic cells that bind ATP and accepts its γ phosphate in a covalent Asp-P bond. Both phospho- and dephospho-forms of the protein exist in two major conformational states E_1 and E_2 with different affinities and orientation of cation binding sites and ATP driven cation pumping is blocked by micromolar concentrations of vanadate (For reviews see DeMeis,1981; Glynn, 1985; Jørgensen, 1982, 1986; Kyte, 1981). The intermediary steps of the pump reactions and their relationship to cation translocation have been examined in detail, particularly for the Na,K-pump and Ca-pump proteins, but our knowledge does not allow construction of detailed molecular models of cation pumping.

During the last 3-4 years, a wealth of structural information about the cation pump proteins has appeared from sequencing cloned cDNA and identification of genes. Several isoforms of the genes of α-subunit of Na,K-pump were identified in the human and rat genomes (Sverdlov et al., 1987; Shull and Lingrel, 1987) and the sequences of α-subunit (Kawakami et al., 1985, 1986a; Ovchinnikov et al. 1986; Shull et al., 1985, 1986a) and β-subunit (Brown et al., 1987; Kawakami et al., 1986b; Ovchinnikov et al., 1986; Shull et al., 1986b) were deduced from cDNA of mRNA from a variety of tissues in piscine and mammalian species. A similar intensity of work in adjacent fields provided the sequences of slow and fast twitch Ca-pump from sarcoplasmic reticulum (Brandl et al., 1986) and H,K-pump from stomach mucosa (Shull and Lingrel, 1986). In addition the gene sequences are available for K-pumps (Hesse et al., 1984; Solioz et al., 1987) and H-pumps (Addison, 1986; Serrano et al., 1986) from

microorganisms. As paradigm for interpretation of this information the combined X-ray crystallographic structure at high resolution (<3 Å) and aminoacid sequences are only available for a few bacterial membrane proteins (Diesenhofer et al., 1985). Crystals of the proteins of membrane bound Na,K-pump (Hebert et al., 1982), Ca-pump (Taylor et al., 1986) and H,K-pump (Rabon et al., 1986) can be prepared by incubation in vanadate solution, but only low resolution (>20-25 Å) three dimensional models can be constructed on this basis.

In this article organization of the αß-unit of Na,K-ATPase in the membrane and the molecular mechanism of Na,K-transport are examined in light of the new information about membrane protein structure. A main purpose is to assign residues involved in formation of nucleotide and cation binding sites and E_1-E_2 transition to their position in the sequence and to explain organization of cation pathways and the relationship of conformational changes to translocation of cations across the membrane. Another problem that must be resolved before meaningful molecular models can be constructed is the question of subunit structure.

PROTOMERIC αß-UNIT AS MINIMUM FUNCTIONAL UNIT FOR LIGAND BINDING AND FORMATION OF CATION PATHWAY.

Recent progress in characterization of soluble Na,K-ATPase suggests that the αß-unit provides structural basis for formation of cation pathways. Protomer αß-unit of Na,K-ATPase is defined as the minimum functional units required for all aspects of the reaction cycle including E_1-E_2 and E_1P-E_2P transitions (Jørgensen and Andersen, 1986) and occlusion of Na and Rb/K (Vilsen et al., 1987, and this volume). Sedimentation coefficients are 6-7 S for protomeric αß-unit of Na,K-ATPase and molecular weights of the soluble enzymes as determined by sedimentation equilibrium and sedimentation velocity studies agree with that calculated from aminoacid composition within +/- 10% (147,000 for αß-unit of Na,K-ATPase). Similar molecular weights are obtained by high resolution gel chromatography combined with low angle laser light scattering of active Na,K-ATPase (Hayashi et al., 1987). These data show that the αß-unit forms the minimum functional unit for Na,K-ATPase activity and formation of the cation pathway. It is important to note that the αß-unit also is the minimum asymmetric unit in membrane crystals (Hebert et al., 1982).

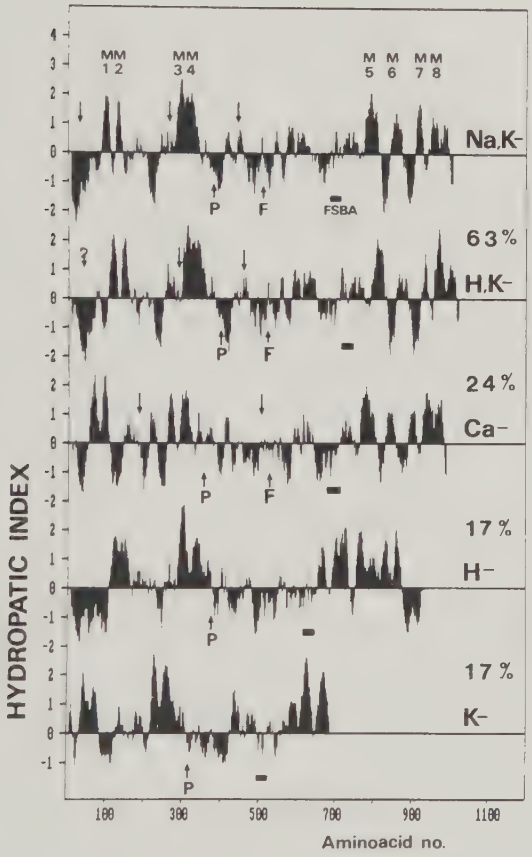

Figure 1. Hydroplots using a window of 19 residues of Na,K- (Kawakami et al., 1986), H,K- (Shull and Lingrel, 1986), Ca- (Brandl et al., 1986), H- (Serrano et al., 1986), and K- (Hesse et al., 1984) pump proteins with overall homology (%) in alignment with α-subunit of human Na,K-ATPase shown to the right. M1-M8 show positions of presumptive transmembrane helices. P is the phosphorylated residue, F the position for covalent insertion of FITC (Karlish, 1980), ▬ marks the segment labeled with FSBA (Ohta et al., 1986). The sequences of these segments are shown in Table I. Vertical arrows mark the positions of primary tryptic cleavage sites. Computer analysis using "Genepro", Riverside Scientific, Washington, Seattle with the help of Dr. T.E. Petersen.

EVOLUTION AND GENE STRUCTURE OF Na,K-PUMP PROTEIN

Comparison of α-subunit of Na,K-ATPase with H,K-, Ca-, H- and K-pump proteins show that overall sequence homology is moderate, 17-24% except for 63% between H,K-ATPase and Na,K-ATPase, while tertiary structure is remarkably similar with respect to localization of transmembrane segments relative to domains involved in ATP binding and phosphorylation, Fig. 1. Homologies are strongest in segments connecting transmembrane helices with phosphorylation site and nucleotide binding area, while there is little or no homology between the aminoacid sequences of N-termini, transmembrane helices, and presumptive cation binding areas in Na,K-ATPase and H,K-ATPase on one hand and Ca-ATPase, H-ATPase, and K-ATPase on the other hand. In accordance with the needs for cation specificity, divergence of cation pump genes may have consisted in recombination of exons involved in recognition, binding of cation and formation of transcellular pathways, while exons coding for domains involved in nucleotide binding, phosphorylation and energy transduction are ancient and best conserved in the pump genomes.

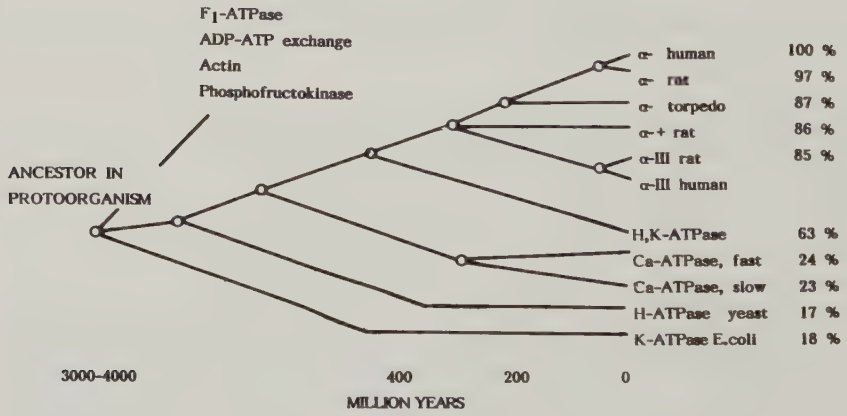

Figure 2. Evolutionary tree for cation pump proteins with overall homologies relative to α-subunit of Na,K-ATPase shown to the right. Sequence homologies and references are shown in Table I. Time scales are based on information about divergence of filetic lines (Dayhoff et al., 1978).

An evolutionary tree can be drawn on basis of the information in mRNA or genes of cation pump proteins and deduced aminoacid sequences, Fig. 2. The ancestor was probably a proton pump, but the properties of the protoorganism existing 3-4 billion years ago (Dayhoff et al., 1978) are not known.

GENES AND ISOFORMS OF Na,K-ATPase.

The first impression of the genes of the Na,K-pump is one of diversity and complexity with at least 5 α-subunit genes of 20-25 kb and multiple introns (Sverdlov et al., 1987; Shull and Lingrel, 1987). At least 3 mRNA isoforms of α-subunit are expressed in rat brain (Shull et al., 1986). From the available information about the distribution and function of isoforms of α-subunit of Na,K-ATPase (Sweadner, 1985; Young and Lingrel, 1987) it is already obvious that detailed analysis of the expression of isoforms using specific [^{32}P]-cDNA probes will reveal important new physiological and pathophysiological aspects of cation pump function in several tissues.

The catalytic properties of the α isoform are well known from studies on purified Na,K-ATPase from kidney (Jørgensen, 1982). In whole kidney the ratio of α to α+ isoform is >280:1 (Young and Lingrel, 1987) and only the α isoform seems to be present in thick ascending limb of Henle. The small amounts of α+ mRNA may be localized to cortical collecting ducts where Na,K-ATPase is more sensitive to digitalis. It is difficult to discern the properties of α+ and α-III isoforms since both forms are abundant in brain (Sweadner, 1985; Young and Lingrel, 1987).

Clarification of DNA structure and mechanisms behind expression of isoforms is important for understanding organization of the protein in the membrane and physiological pump functions. Partial sequencing of the α-III gene shows that it is a large, 20-25 kb, and complex structure with 22 introns separating the exons, Fig. 3. By comparison, only 4 introns are found in the gene of H-ATPase in Neurospora Crassa (Addison, 1986) and the gene of H-ATPase in yeast has no introns (Serrano et al., 1986).

Introns form more than 80% of α-subunit gene sequences and their positions may correspond to functional subdivisions of the protein as shown in Fig. 3. Thus, several transmembrane segments (M2, M3, M4, M6, M7, and M8) are bordered by introns. Introns also

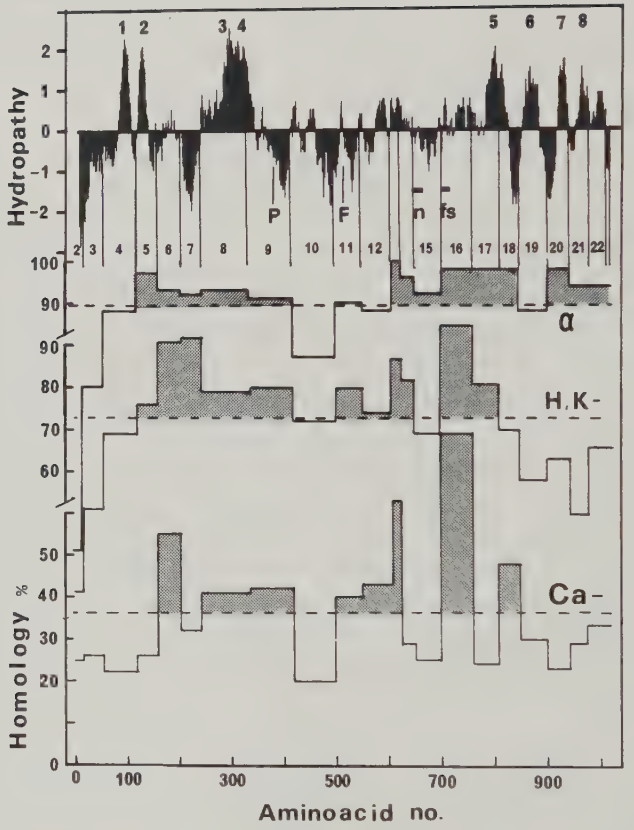

Figure 3. Position of introns in the gene of α-III isoform (Sverdlov et al., 1987) of Na,K-ATPase relative to a hydroplot of the aminoacid sequence. Exons correspond to the numbers and intron positions are marked with vertical lines. M1-M8 refer to positions of transmembrane helices. Below the hydroplot the dotted lines show overall homologies and the columns show homologies of each exon in α-III isoform with α isoform (Shull et al., 1986a), H,K-ATPase (Shull and Lingrel, 1986) and Ca-ATPase (Brandl et al., 1986). Since this figure is concerned with functional aspects, homologies include conservative substitutions (A-G, D-E, L-I, L-V, I-V, R-K, Q-N, S-T, Y-F).

separate exons corresponding to domains involved in formation of ATP binding area (exons 9, 11, 15 and 16). Other exons (no. 1-3, 10, 12,and 19) relate to domains in which homologies between α-isoforms are well below overall homology (85-86%). Calculation of relative homologies within exons is of interest for identifying specific functional regions. E.g. in exon 7 and 17, α, α+, and αIII isoforms show homology mutually and with H,K-ATPase, but not with Ca-ATPase suggesting that segments contributing to K-sites may be located here. Exons 20-22 may correspond to domains that are specific for the Na,K-pump like Na-sites, ouabain site, or interaction with ß-subunit, since they show high mutual homology between α isoforms, while homology with H,K-ATPase and Ca-ATPase is well below the average value.

ORGANIZATION OF αß-UNIT IN THE MEMBRANE

Understanding membrane organization requires that information derived from aminoacid sequences is reconciled with results of proteolytic cleavage and specific chemical labeling of protein from the membrane surfaces and the hydrophobic interior. The membrane organization from these data should correlate with mass distribution between extra- and intramembranous domains as estimated from models of membrane crystals.

Crystallization in the Membrane

Exploiting the very dense packing of asymmetrically oriented protein in purified Na,K-ATPase a method was developed for crystallization in the membrane by incubation in vanadate medium (Hebert et al., 1982, 1985). This also induces formation of crystalline arrays of Ca-ATPase in sarcoplasmic reticulum (Taylor et al., 1986) and preparations of H,K-ATPase (Rabon et al., 1986). In models of Na,K-ATPase and Ca-ATPase the cytoplasmic protrusions of the proteins are remarkably similar. A notable difference is a 10-20 Å protrusion on the extracellular surface of the model for Na,K-ATPase, while the Ca-ATPase model has a smooth extracytoplasmic surface. Superposition of a model of ß-subunit crystals formed in Mg-solution shows that the ß-subunit forms the extracellular protrusion (Ovchinnikov et al., 1986). The intramembranous portion estimated from this αß-unit model is 30-40% of the total mass of the three dimensional model of the αß-unit.

Mass of Intramembrane Protein

Calculation of mass distribution of the model in Fig. 4 with 8 transmembrane segments in α-subunit and 1 in ß-subunit shows that 27% of αß-unit mass is extracellular, 58% is exposed on the cytoplasmic surface and only 15% of αß-unit mass is inside the membrane. This mass of 8+1 transmembrane segments is much less

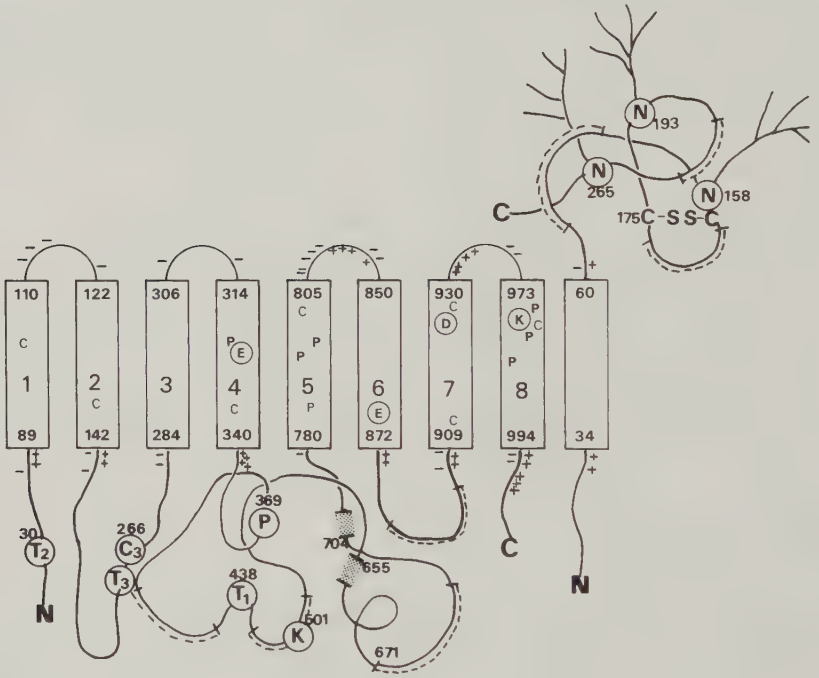

Figure 4. Disposition of αß-unit in the membrane, based on sequence information (Kawakami et al., 1986a, 1986b), labeling with FSBA (Ohta et al., 1986a), and selective proteolytic digestion of α-subunit (Jørgensen and Collins, 1986). Model for ß-subunit is based on sequencing of surface peptides and identification of an S-S bridge (Ohta et al., 1986b). T_1, T_2, T_3, and C_3 show location of proteolytic splits. N are glycosylated asparagins in the ß-subunit. (+) and (-) indicate all extracellular charged residues and charges within the first 10 residues from the transmembrane segments at the cytoplasmic face. (▭) shows FSBA labeling and (——) segments located at the protein surface.

than the mass of intramembrane protein estimated from the crystal model. An alternative estimate of mass distribution can be obtained by proteolysis. Extensive digestion with excess trypsin or unspecific proteases shows that 40-50% of the protein of Na,K-ATPase (Karlish et al., 1977) is protected by lipid or secondary structures that are resistant to proteolysis. With this in mind it is appropriate to consider the possibility that the cation pumps possess intramembrane protein structures in addition to those of predicted transmembrane helices.

Intramembrane Structure of α-Subunit of Na,K-ATPase

The presence of four transmembrane segments in the N-terminal half of the α-subunit was predicted from the results of controlled tryptic cleavage combined with selective chemical labeling from the membrane surfaces and from the hydrophobic interior. In contrast, neither chemical labeling experiments nor hydroplot analysis lead to a decision as to whether 2, 4, or 6 transmembrane segments are formed by the C-terminal part of the α-subunit (Res.779-1016) and sidedness of C-terminus remain uncertain.

In the model of αβ-unit of Na,K-ATPase in Fig. 4, the transmembrane segments M1, M2, M3, M4, M5 have a hydrophatic index of >2.0 and three segments M6, M7, and M8 have a peak index of >1.5 using a window of 19 residues for the hydroplot. These segments consist of 21-25 aminoacid residues with overrepresentation of hydrophobic residues FILVWY, but also of P and C. This may suggest that S-S bridge formation is part of stabilizing intramembrane structures. The excess of prolins may suggest that helices are kinked or that they are not correctly assigned α-helical structures. Few basic or acidic sidechains are found in the transmembrane segments in Fig. 4, but the segments carry charged residues close to their cytoplasmic ends that may react with headgroups of lipids to stabilize the structure in the membrane (Brotherus et al., 1980).

Possible candidates for additional intramembrane structures in α-subunit are relatively hydrophobic segments with high β-sheet propensity in the cytoplasmic domains, e.g. segments 175-202, 242-260, 410-430, and 560-590 in α-subunit. These segments alternate with α-helices and may form flexible structures that contribute to cation binding and E_1-E_2 transition.

TABLE I

Sequence homologies of nucleotide binding and phosphorylation domains

a) Phosphorylation domain

Res.no																
							*									
367	T	S	T	I	C	S	D	K	T	G	T	L	T	Q	N	R	M	Na,K, α
379	T	S	V	I	C	S	D	K	T	G	T	L	T	Q	N	R	M	H,K-
345	T	S	V	I	C	S	D	K	T	G	T	L	T	T	N	Q	M	Ca-
372	V	E	I	L	C	S	D	K	T	G	T	L	T	K	N	K	L	H-, yeast
301	V	D	V	L	L	L	D	K	T	G	T	I	T	L	G	N	R	KdpB
273	L	D	V	I	M	L	D	K	T	G	T	L	T	Q	G	K	F	K-, S.f.
280	Q	E	R	I	T	S	T	K	T	G	S	I	T	S	V	Q	A	F1-, ß, coli
293	Q	E	R	I	T	T	T	K	K	G	S	I	T	S	V	Q	A	F1-, ß, bov.

b) FITC reactive region

								*										
496	P	Q	H	L	L	V	M	K	G	A	P	E	R	I	L	D	R	C	S	S	Na,K, α
510	P	R	H	L	L	V	M	K	G	A	P	E	R	V	L	E	R	C	S	S	H,K-
508	V	G	N	K	M	F	V	K	G	A	P	E	G	V	I	D	R	C	N	Y	Ca-
					M	Y	S	K	G	A	S	E	I	I	L	R					Ca-p.m.
467	G	E	R	I	V	C	V	K	G	A	P	L	S	A	L	K	T	V	E	E	H-, yeast
467	G	E	R	I	T	C	V	K	G	A	P	L	F	V	L	K	T	V	E	E	H-, neurosp.
388	I	D	N	R	M	I	R	K	G	S	V	D	A	I	R	R	H	V	E	A	KdpB

c) Nucleotide binding region

543	L	G	E	R	V	-	L	G	F	C	H	L	F	L	P	D	E	Q	F	P	Na,K-, α
613	L	K	C	R	T	-	A	G	I	R	V	I	M	V	T	G	D	H	P	I	H,K-
611	Q	L	C	R	D	-	A	G	I	R	V	I	M	I	T	G	D	N	K	G	Ca-
544	C	E	A	K	T	-	L	G	L	S	I	K	M	L	T	G	D	A	V	G	H-, neurosp.
545	S	E	A	R	H	-	L	G	L	R	V	K	M	L	T	G	D	A	V	G	H-, yeast
243	E	Y	F	R	D	Q	E	G	Q	D	V	L	L	F	I	D	N	I	F	R	F1-, ß, bov.
267	E	Y	F	R	D	-	R	G	E	D	A	L	I	I	Y	D	D	L	S	K	F1-, α, coli
277	V	L	-	R	G	N	G	G	A	F	V	L	V	L	Y	D	E	I	K	K	ATP-ADP exch.
104	E	F	E	R	K	-	I	G	Q	P	T	L	L	L	Y	V	D	A	G	P	Aden. kin.
87	E	Q	L	K	K	-	H	G	I	Q	G	L	V	V	I	G	G	D	G	S	Phosphofr.

d) Segment binding FSBA and Cl-ATP in Na,K-ATPase

									*									
703	Q	G	A	I	V	A	V	T	G	D	G	V	N	D	S	P	A	L	K	K	Na,K-, α
717	L	G	A	I	V	A	V	T	G	D	G	V	N	D	S	P	A	L	K	K	H,K-
694	Y	D	E	I	T	A	M	T	G	D	G	V	N	D	A	P	A	L	K	K	Ca-
625	R	G	Y	L	V	A	M	T	G	D	G	V	N	D	A	P	S	L	K	K	H-, neurosp.
625	R	G	Y	L	V	A	M	T	G	D	G	V	N	D	A	P	S	L	K	K	H-, yeast
509	E	G	R	L	V	A	M	T	G	D	G	T	N	D	A	P	A	L	A	Q	KdpB
467	Q	G	K	K	V	T	M	V	G	D	G	I	N	D	A	P	S	L	A	R	K-S.f.

References to sequences: Na,K-ATPase, α (Kawakami et al., 1986), H,K-ATPase (Shull et al., 1986), Ca-ATPase (Brandl et al., 1986), Ca-ATPase, plasma membr. (Filoteo et al., 1987), H-ATPase, yeast (Serrano et al., 1986), H-ATPase, Neurospora (Addison, 1986), KdpB (Hesse et al., 1984), K-ATPase, Str. faecalis (Solioz et al., 1987), F1-ATPase, ß, coli and bovine, ADP-ATP exchange, phosphofructokinase (Walker et al., 1982), adenylate kinase (Fry et al., 1985).

Sites for Nucleotide Binding and Phosphorylation in Na,K-ATPase

A characteristic structural feature of the α-subunit is a cytoplasmic protrusion with approximate dimensions 45 x 65 Å in the plane of the membrane and a length of 50-60 Å in the plane perpendicular to the membrane. As illustrated in Fig. 4, transmembrane segments separate the protrusion into domains and selective cleavage and chemical labeling established structure-function correlates for some of these domains. The N-terminus is attached to M1 and is important for control of E_1-E_2 transition and the rate of Na,K-pumping. The second cytoplasmic domain (142-284 in α-subunit) between M-2 and M-3 exposes bonds to cleavage in E_1-forms. The bulk of the protrusion is formed by the large central domain (Res. 340-780 in α-subunit) that forms sites for ATP binding and phosphorylation.

Studies of other ATP binding proteins show that functional residues are contributed by regions of different protein sequences and secondary structure. An indication of the functional groups required to form a nucleotide site may be obtained from comparison with sites in dehydrogenases, phospho-fructokinase and in particular, adenylate kinase (Fry et al., 1985). An important feature is a hydrophobic pocket for accommodation of the adenine and ribose moieties which is formed by Ile, Val, His and Leu residues. The triphosphate moiety is flanked by a hydrophobic strand of parallel ß-pleated sheet terminated by Asp (Fry et al., 1985). Table I c) shows that such a segment in adenylate kinase and ß-subunit of F1-ATPase shows homology with α-subunit and other cation pump proteins. The N-terminal part of this segment is rich in glycins and may form a flexible loop that may undergo conformational transitions related to altered affinity of the site or to reallocation of catalytic groups.

Labeling with ATP analogues provide evidence for contribution from additional segments in α-subunit of Na,K-ATPase. The first indication came from ATP sensitive covalent insertion of FITC into Lys-501 in α-subunit (Karlish, 1980). Two peptide segments (655-664 and 704-722) are labeled by FSBA (Ohta et al., 1986) 5-(p-fluorosulfonyl)-benzoyl-adenosine and γ-[4-(N-2-chlorethyl-N-methylamino)]benzoylamid-ATP (ClR-ATP) that inserts covalently in Asp-710 in the 704-722 segment (Ovchinnikov et al., 1986). These ATP analogues may react with charged residues coordinating phosphate moieties in ATP.

Surface peptides released from the central domain of α-subunit by tryptic cleavage have also been sequenced (Ohta et al., 1986) and a provisional arrangement of the ATP binding domain in the

cytoplasmic protrusion has been drawn as in Fig. 4 without attempts at illustrating molecular detail of the binding site.

Structural Changes in Cytoplasmic Domain Accompanying E_1-E_2 Transitions

Exposure and protection of bonds on the surface of the cytoplasmic protrusion gave unequivocal evidence for structural changes in α-subunit accompanying E_1-E_2 transition in Na,K-ATPase. Definition of E_1 and E_2 conformations of α-subunit of Na,K-ATPase involves identification of cleavage points in the protein as well as association of cleavage with different rates of inactivation of Na,K-ATPase and K-phosphatase activities (Jørgensen, 1975, 1977).

This provided a shortcut to identification of residues involved in E_1-E_2 transition (Jørgensen and Collins, 1986) and conformation dependent cleavage patterns have been detected in Ca-ATPase (Andersen et al., 1986). The transition involves residues in ATP binding and phosphorylation domains and it is transmitted to the extra-cytoplasmic surface with changes in binding affinity for cations and ouabain. In addition to bonds exposed to proteolysis, the transition involves tryptophans, sulfhydryl groups, protonizable groups and residues binding FITC and other fluorescent ligands. (See previous reviews for details (Glynn, 1985; Jørgensen, 1982)).

Transposition of Mass Accompanying E_1-E_2 Transition

Structural studies and hydrophobic labeling suggest a deeper immersion of peptide segments in the bilayer in E_2-forms than in E_1-forms. The hydrophobic photoactivable [^{125}I]-iodonaphtylazide labels E_2-forms 10-25% more than E_1-forms of Na,K-ATPase (Karlish et al., 1977). Preferential hydrophobic labelling of E_2 forms of Ca-ATPase with TID is located in the A1 fragment (Res. 199-505) with 70% higher labeling than in other segments of Ca-ATPase. The A1 fragment also contains the phosphorylated aspartyl acid residue. The difference between hydrophobic labelling of E_1- and E_2-forms corresponds to movement of a minimum of 38 residues into the bilayer (Andersen et al., 1986).

Cation Binding Sites and Conformation of Occluded Forms

It has not been possible to assign Na^+ and K^+ binding sites to residues in the aminoacid sequences and it is not known if they are part of the intramembrane portion of the pump protein. Understanding the relationship between occlusion-deocclusion and E_1-E_2 transition may be a key to solving the transport mechanism. Rb/K are occluded in an E_2[K] form (Glynn, 1985) and it is probable than an E_1[K] occluded form is stabilized after chymotryptic cleavage (Jørgensen and Petersen, 1985). Occlusion of Na was demonstrated in the E_1P[Na] form after chymotryptic cleavage or treatment with NEM (Glynn, 1985) or oligomycin (Esmann and Skou, 1985).

Using CrATP as phosphorylating substrate Vilsen et al. (1987) measured directly values of Na occlusion as high as 11 nmol/mg protein, Fig. 5. In selected purified preparations of Na,K-ATPase incorporation of ^{32}P from [$\gamma^{32}P$]-CrATP amounts to 6 nmol EP/mg

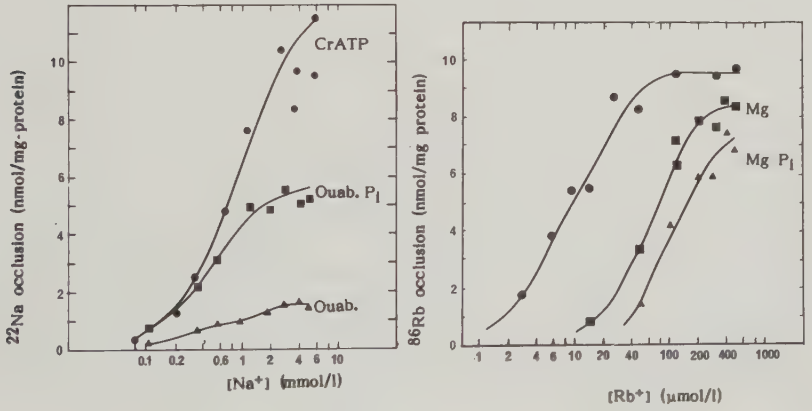

Figure 5. Occlusion of ^{22}Na and ^{86}Rb in membrane-bound Na,K-ATPase. Procedures as described in Vilsen et al. (1987 and this volume). CrATP: incubation with increasing concentrations of Na for 3 h at 20° C with 0.5 mM CrATP, 1 mM $MgCl_2$, in 10 mM Tris, 0.1 mM EDTA, pH 7.5. Ouabain: incubation in the same buffer for 15 min at 20° C with 1 mM ouabain, 1 mM $MgCl_2$ with or without 1 mM P_i-Tris. For Rb-occlusion incubation was for 15 min at 20° C without or with addition of 1 mM $MgCl_2$ and 1 mM P_i-Tris.

at 20° C without or with addition of 1 mM $MgCl_2$ and 1 mM P_i-Tris.

protein. This yields a stoichiometry of 1.8 Na/EP, but appropriate extrapolation for unsaturation of binding sites corrects this value to 2.7 Na/EP. Previously, maximum values of only 2-3 nmol occluded Na/mg protein were measured after pretreating Na,K-ATPase with chymotrypsin, NEM or oligomycin. Phosphorylation from CrATP stabilizes a Na-occluded conformation CrADP-E_1P[Na], reminiscent of the E_1P form (Pauls et al., 1986). Examination of tryptic and chymotryptic cleavage patterns confirm that the α-subunit is stabilized in an E_1-form (unpublished). In Fig. 5 occlusion of Na in CrADP-E_1P[Na] is compared with occlusion of Na in complexes with ouabain. In Fig. 5 is seen that the capacity for occlusion of Na is low in presence of Mg and ouabain, but that it is increased to 5-6 nmol/mg protein in presence of 1 mmol/l phosphate without changing the apparent affinity for Na. In the E_2-form the phosphorylated residue may thus be necessary for occlusion or directly involved in coordinating the cation. The capacity for Na occlusion by the Ouab-E_2P[Na] complex seems to be lower than that of the CrADP-E_1P[Na] complex. It is therefore possible that the transition from E_1P to E_2P involves the release of one of the occluded Na ions.

Demonstration of Na occlusion in both E_1P and E_2P phosphoforms poses restrictions on the structural models for transport. The straightforward interpretation of the data is that occlusion of Na is a condition for transition from E_1P to E_2P and that a change in coordination of 369-Asp-phosphate may trigger this transition. The important movement of the protein accompanying E_1-E_2 transition may thus consist of a change in orientation of the coordinated complex from orientation towards the near side to orientation towards the far side. Rb and Na compete for occlusion in complexes of Na,K-ATPase with ouabain. This observation is in agreement with the notion that a single set of cation sites may bind and occlude either Na^+ or Rb^+ (Forbush, 1987a).

The Rb occlusion data in Fig. 5 extrapolates to about the same total capacity of 9-10 nmoles/mg protein, but phosphorylation from P_i drastically reduces apparent affinity for Rb; from $K_{0.5}$ 7.8 μmol/l to 365 μmol/l, i.e. by 47-fold. This antagonism is in agreement with the notion that Rb/K displaces P_i by increasing the rate of hydrolysis of the phosphate bond. In contrast phosphorylation has little effect on the apparent affinity for Na.

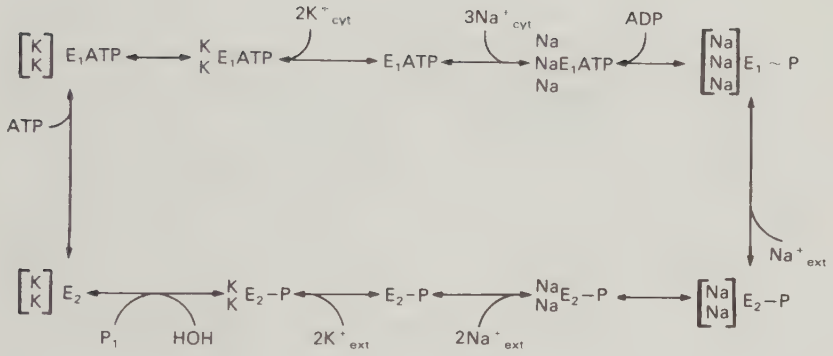

Figure 6. E_1-E_2 reaction cycle of the Na,K-pump with ping-pong sequential cation translocation. The scheme is based on the demonstration of 4 major occluded conformations, but it otherwise similar to the Post-Albers scheme. The phosphoforms occlude Na and dephosphoforms occlude K or Rb. Na and K without brackets are cations bound to an open form such that they can exchange with medium cation. [Na] or [K] within brackets are occluded and prevented from exchanging with medium cations. It is proposed that release of Na_{ext} accompanies transition from E_1P[Na] to E_2P[Na] since capacity for occlusion of Na in the ouabain stabilized form is lower than after incubation with CrATP (Fig. 5).

Model for Occlusion and Translocation of Na^+ and K^+

The demonstration of occluded E_1- and E_2-forms in combination with a single set of sites for binding of Na^+ or Rb^+ may be understood in terms of a moving pore model (Cf. Tonomura, 1986) in which the steps of ion binding and occlusion are distinct from the E_1-E_2 transition. In this model cation binding sites consist of electrophilic carbonyl groups that are located in a cavity to accommodate the cations inside the protein. Transition from an open to the occluded configuration involves only limited changes in

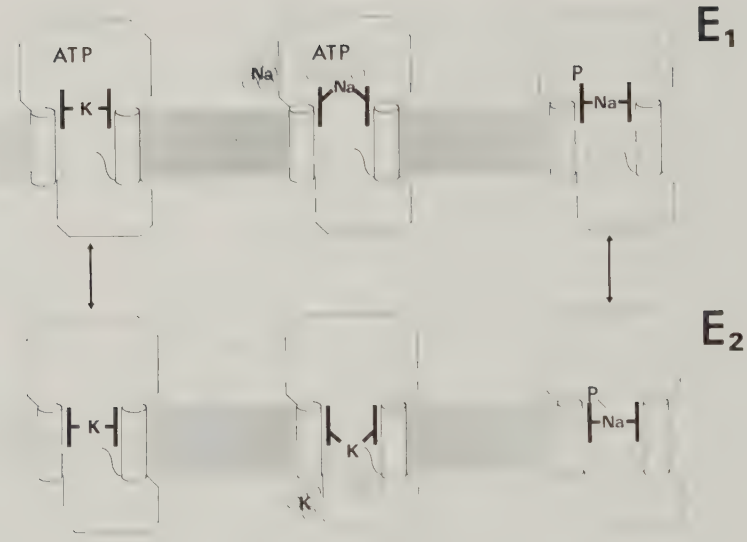

Figure 7. A structure model for formation of cation pathway through the αß-unit of Na,K-ATPase with reference to the reaction scheme in Fig. 6. The αß-unit possess cytoplasmic (α-subunit) and extracellular protrusions (ß-subunit) and an intramembrane portion. The cylinders indicate a sheet of 8+1 transmembrane helices. Inside this sheet, a functional core organized in amphipatic helices or ß-sheet structures forms the cation binding region. Motion of the cation coordinating groups constitutes an essential part of the E_1-E_2 transition. This transition effects transfer of occluded cations between a position near the intracellular aspect of the pump protein to a position near the extracellular mouth of the cation pathway. The E_1-E_2 transition involves transposition of mass from relatively hydrophilic to hydrophobic environments. This may be achieved by removing charges and exposing hydrophobic segments to the outer ring of hydrophobic transmembrane helices of the αß-unit.

conformation of carbonyl residues and a substitution of solvent molecules from the inner coordination sphere of the cation. Dehydration of Na^+ takes place on the cytoplasmic surface and after coordination of the cation, the carbonyl groups rearrange from open to occluded form. An essential part of the E_1-E_2 transition is the movement of the residues possessing the coordinating carbonyl groups from the bottom of the cytoplasmic mouth to the bottom of the extracellular mouth of the cation pathway. At the extracellular mouth of the cation pathway the site shifts from closed to open configuration and Na^+ is rehydrated and released. The same set of coordinating groups, except phosphate, bind K^+ from the outside. The mouth of the cation pathway is similar to the mouth of the K-channel which has the function of dehydrating K^+ before it passes through the narrow part of the channel (Cf. Stein, 1987). This moving pore model with a cleft for passage of dehydrated cations in a coordinated complex with carbonyl groups may be seen as alternative to a combination of gates or ionophore functions in combination with a hydrated pore (Cf. Brandl et al., 1986).

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OVERVIEW:

THREE-DIMENSIONAL STRUCTURE AND TOPOGRAPHY OF MEMBRANE BOUND Na,K-ATPase

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INTRODUCTION

Analysis of isolated membranes by high resolution electron microscopy after negative staining and freeze-fracture provides information about the structure of the protein molecules and their organization in the membranes. Such information may include dimensions and frequency of the molecules and the interrelationships of their subunits. If the molecules are arranged in two-dimensional crystalline arrays the electron micrographs can be analyzed with Fourier methods and image processing and additional information is obtained about the projection structure of the protein. Furthermore, analysis of tilted crystals allows reconstruction of the three-dimensional structure of the molecule.

Na,K-ATPase is suitable for analysis by electron microscopy after its isolation in membrane-bound form. In Na,K-ATPase membranes, isolated in the presence of SDS and ATP (Jørgensen, 1974), the enzyme protein has a random distribution. However, when the purified enzyme is incubated in a medium containing vanadate or phosphate and magnesium we found that the membrane-bound Na,K-ATPase forms two-dimensional crystals (Skriver et al., 1981). This demonstration opened the possibility for further studies of Na,K-ATPase with electron microscopy and image processing. As results average projection structures (Hebert et al., 1982) as well as a three-dimensional structure of the enzyme protein were obtained (Hebert et al., 1985a; Ovchinnikov et al., 1985). More recently additional aspects of the three-dimensional structure of renal Na,K-ATPase, as observed by electron microscopy, has been presented both with respect to dimeric (Mohraz et al., 1987) and monomeric crystals (Hebert et al., this volume) and the β-subunit (Demin et al., this volume).

In this overview we will summarize and discuss some of the structural observations on Na,K-ATPase as observed by electron microscopy and in particular observations derived from the analysis of membrane crystals.

NON-CRYSTALLIZED ENZYME

Following negative staining with phosphotungstic acid or uranyl acetate purified Na,K-ATPase membranes are disc-shaped and exhibit a uniform population of surface particles with a frequency of 12,500 particles/μm^2 membrane surface (Maunsbach and Jørgensen, 1974; Deguchi et al., 1977). The particles are not randomly distributed over the membrane surfaces but arranged in irregular clusters or strands. On the basis of the frequency and size of the particles as related to the known enzymatic activity and chemical composition of the isolated membranes we concluded that each surface particle represents one $\alpha\beta$-unit (protomer) of the enzyme. Between the clusters of $\alpha\beta$-units are empty areas of irregular shape which correspond to the lipid bilayer of the isolated membranes. Some isolated membrane fragments are in continuity with elongated narrow projections with particles projected on one or both sides (Vogel et al., 1977; Maunsbach et al., 1982). Such long and undulating cylindrical structures have been interpreted as linear polymers of $(\alpha\beta)_2$ dimers of Na,K-ATPase molecules and suggested to be the result of a secondary aggregation process occurring during the purification procedure (Zampighi et al., 1986).

The distribution of enzyme proteins in isolated membranes can be modified by cross-linking with glutaraldehyde. After exposure of the membranes to low concentrations of glutaraldehyde the surface particles form large clusters. This observation demonstrated that the $\alpha\beta$-units have extensive lateral mobility in the plane of the membrane (Deguchi et al., 1977).

Freeze-fracture electron microscopy has revealed aspects of the organization of the Na,K-ATPase protein within the membrane. Tangentially fractured membranes exhibit intramembrane particles with a diameter of approximately 90 Å on both fracture faces. The particles show an asymmetric distribution between the fracture faces. The particle-rich fracture faces average 5,600 particles/μm^2 whereas the particle-poor fracture faces average 500 particles/μm^2. The combined frequency of particles on the two fracture faces is thus 6,100 particles/μm^2 (Maunsbach et al., 1979). On the basis of their size and frequency each intramembrane particle is interpreted as one $(\alpha\beta)_2$-unit of

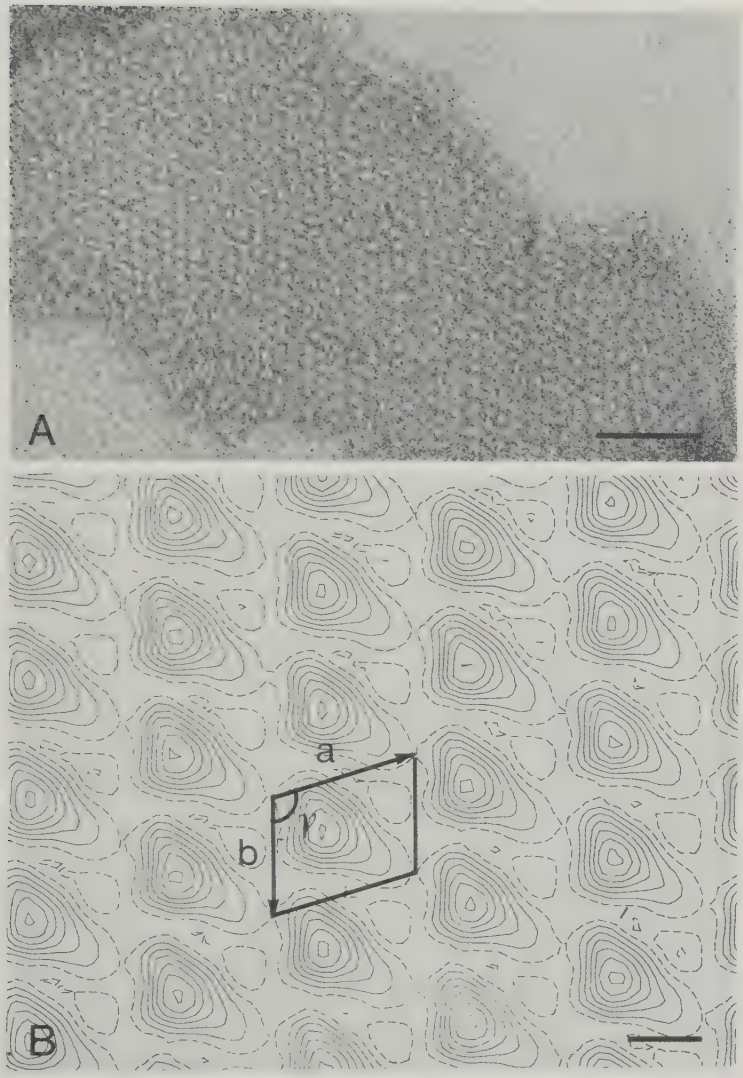

Fig. 1A. Electron micrograph of protomeric two-dimensional crystal of renal Na,K-ATPase. Bar: 500 Å.

Fig. 1B. Computer-reconstructed image from a similar crystal. The repeating units drawn with unbroken contour lines represent enzyme protein and correspond to the protomer of the enzyme and the dashed lines outline the uranyl acetate used for negative staining. Unit cell dimensions are: a = 69 Å, b = 53 Å, $\gamma = 105^{o}$. Bar: 30 Å (modified from Hebert et al., 1982).

Na,K-ATPase (Deguchi et al., 1977). Freeze-fracture followed by rotary replication provided additional evidence for a dimeric structure of some of the intramembrane particles. Thus, fracture faces which were rotary replicated with a thin layer of platinum at low angle exhibited many particles which appeared to consist of two approximately equal subunits (Maunsbach et al., 1979). Freeze-fracture electron microcopy of liposomes reconstituted with purified Na,K-ATPase revealed that the frequency of intramembrane particles correlated with the amount of enzyme used for reconstitution and with the active sodium transport. Thus, the intramembrane particles represent sites of active ion transport (Skriver et al., 1980).

INDUCTION AND ASSEMBLY OF 2-D CRYSTALS

Incubation of isolated Na,K-ATPase membranes with ligands which affect the conformational state of the enzyme protein influences the pattern of surface particles observed by negative staining. In a systematic search for conditions which assemble the enzyme protein in regular crystalline arrays we exposed purified, membrane-bound Na,K-ATPase to a number of combinations of ligands known to interact with Na,K-ATPase. Incubation of purified membrane-bound Na,K-ATPase with vanadate in the presence of magnesium was found to be an efficient method to form two-dimensional crystalline arrays (Skriver et al., 1981).

Computer-averaged projection images of the two-dimensional crystals show the presence of different crystal forms. One form can be assigned two-sided plane group symmetry p1 and contains one protomeric αβ-unit of the enzyme protein in the unit cell (Fig. 1). The unit cell dimensions observed for such protomeric crystals were found to vary slightly in different studies (Table 1). This variation may depend upon the maturity of the crystals.

Crystals with two-sided plane group symmetry p21 appear in two different forms, type I and type II (Figs. 2 and 3). In both forms the projection maps demonstrate that the unit cell contains two strong positive regions, suggesting that two αβ-units occupy one unit cell. The two forms of dimer crystals differ with respect to the dimensions of the b-axis, which is longer in type II crystals (Table 2). In addition the protein units (protomers) have different projection forms in the two types, indicating that they may have different rotations in the unit cells (compare Figs. 2B and 3B). Furthermore the contact regions between the protein units are different in type I and type II crystals.

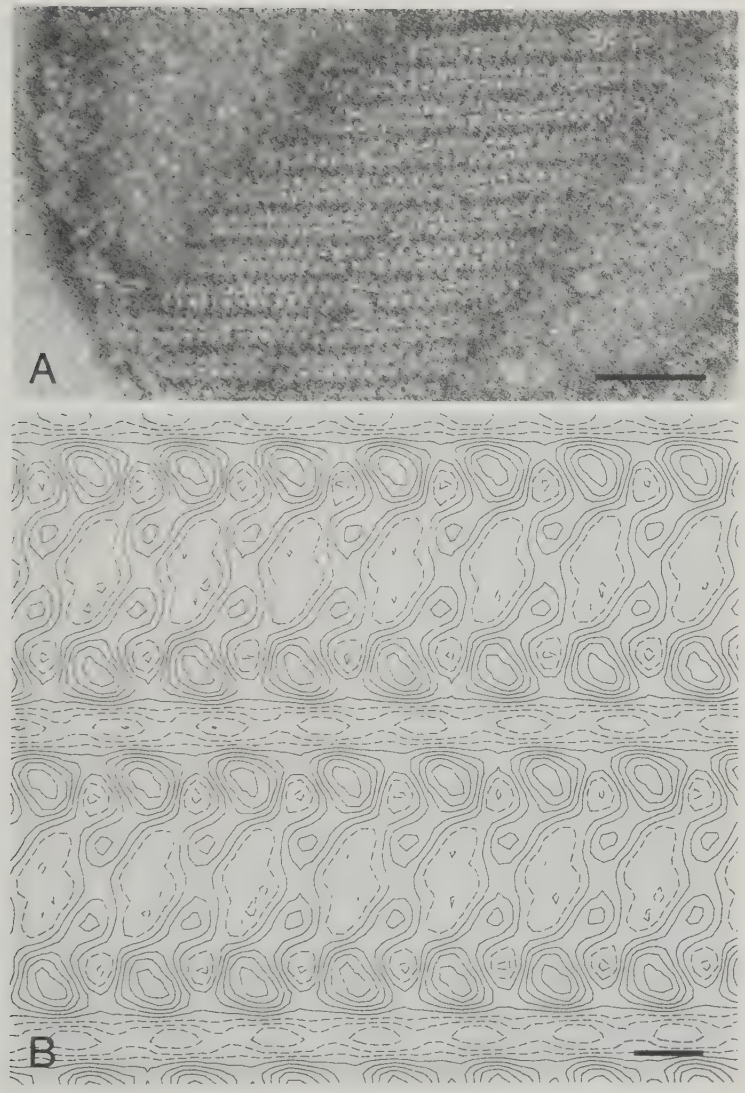

Fig. 2A. Electron micrograph of dimeric two-dimensional crystal of type I. Bar: 500 Å.

Fig. 2B. Computer-reconstructed image from a similar crystal. The unit cell contains two strong positive regions. Each region (unbroken contour lines) is subdivided into one large and one small peak which have been suggested to include the α- and β-subunits, respectively. Bar: 30 Å (modified from Hebert et al., 1982).

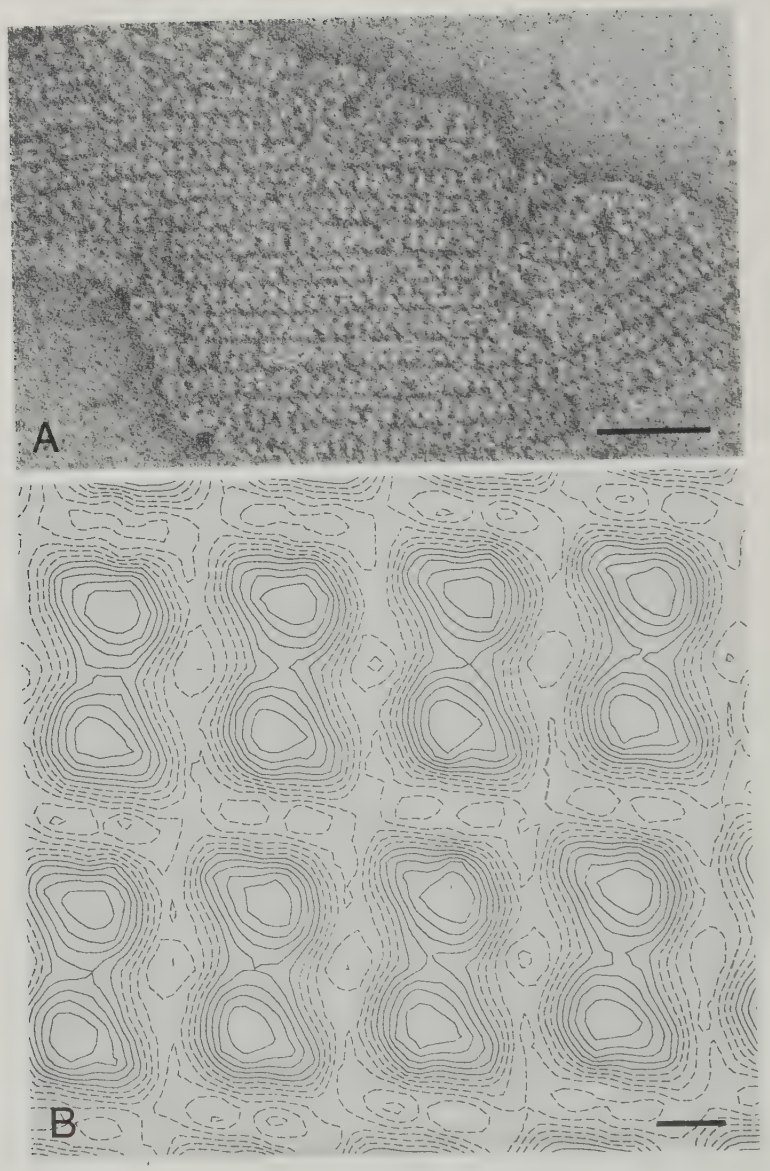

Fig. 3A. Electron micrograph of dimeric two-dimensional crystal of type II. Bar: 500 Å.

Fig. 3B. Computer-reconstructed image from a similar crystal. As in Fig. 2B each unit cell contains two strong positive regions, which have different shapes than in type I crystals. Bar: 30 Å.

TABLE 1. Crystallographic parameters of two-dimensional protomeric crystals of renal Na,K-ATPase with two-sided plane group symmetry p1.

Species	a (Å)	b (Å)	γ (°)	Reference
pig	69	53	105	Hebert et al., 1982
pig	53	51	120	Hebert et al., 1982
pig/dog	59	56	110	Mohraz and Smith, 1984
dog	60	50	100-116	Zampighi et al., 1984
pig	70	59	111	Demin et al., 1984

TABLE 2. Crystallographic parameters of two-dimensional type I and type II dimeric crystals of renal Na,K-ATPase with two-sided plane group symmetry p21.

Species	a (Å)	b (Å)	γ (°)	Reference
Type I				
pig	135	44	101	Hebert et al., 1982
pig/dog	148	55	94	Mohraz and Smith, 1984
pig	166	56	91	Demin et al., 1984
dog	125	55	100-108	Zampighi et al., 1984
dog	144	49	98	Mohraz et al., 1985
rabbit	120	45	107	Hebert et al., 1985b
dog	143,5	49	98	Mohraz et al., 1987
Type II				
pig	123	72	103	Demin et al., 1984
rabbit	126	76	96.5	Hebert et al., 1985a
pig	118	66	108	Ovchinnikov et al., 1985

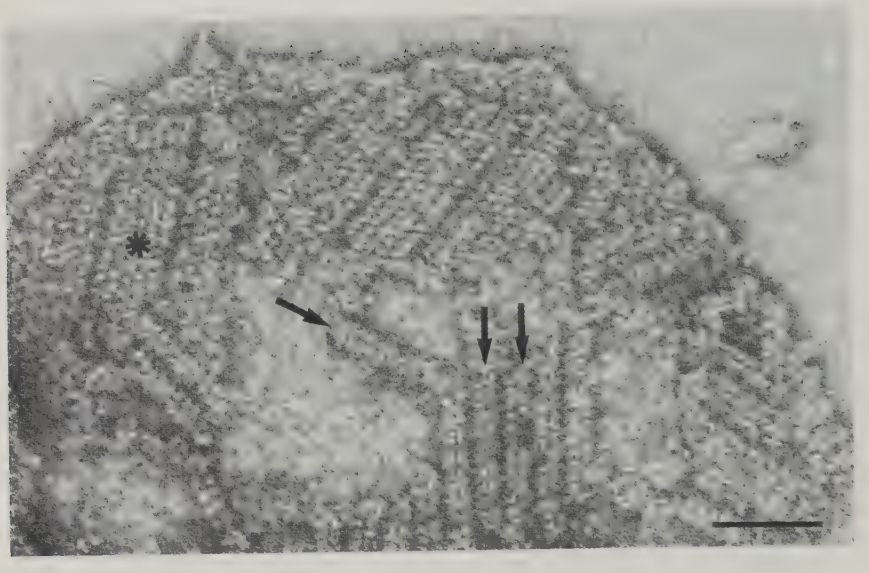

Fig. 4. Linear arrays of Na,K-ATPase dimers (arrows) formed during incubation of purified, membrane-bound enzyme with vanadate in the presence of magnesium. The membrane fragment also shows protomeric crystalline arrays (*). Bar: 500 Å.

During incubation with vanadate both protomeric and dimeric membrane crystals can be observed at the same time in the preparations. During the first hours and days of incubation the largest proportion of crystals are of the dimeric type. Subsequently the frequency of protomeric type crystals increases, while the frequency of dimeric crystals gradually diminishes. By appropriate choice of incubation time it is therefore possible to prepare samples where there is an overweight of either dimeric or protomeric crystals of Na,K-ATPase.

There is no difference in the rate and extent of crystallization when crystals are induced with $NaVO_3$ or NH_4VO_3 (Skriver and Maunsbach, 1983). However, incubation of the enzyme with crystal-inducing ions in presence of phospholipase A_2 seems to enhance the formation of dimeric crystals type I (Mohraz et al., 1985), although we also observe protomeric crystals in these preparations. Addition of 150 mM KCl induces more crystallization centers within each membrane and often results in several

small lattice systems in one membrane fragment (Skriver et al., 1985). Also other ligands than vanadate induce crystalline arrays of the protein in the Na,K-ATPase membranes, although the extent of crystallization varies among the ligands. Phosphate in the presence of magnesium induces crystalline arrays mainly of the dimeric type but protomers are also present. Magnesium in the presence of 0.5 mM EGTA induces membrane crystals at a rate and extent almost similar to that observed in vanadate/magnesium medium (Skriver et al., 1985).

Assembly of crystalline arrays, as followed by electron microscopy after negative staining, is observed within minutes after the start of incubation with vanadate in some membrane fragments. The initial formation of $(\alpha\beta)_2$-units in the assembly of p21 crystals is emphasized by the observation of linear two-rowed arrays or ribbons (Fig. 4). Correlation averaging of such ribbons show pairs of $\alpha\beta$-units with a projection structure similar to that of fully developed p21 crystals (Söderholm et al., 1986). In the center the ribbon has a deep stain filled core surrounded by protein units related by a two-fold axis. The stalk of the protein units are directed away from the core. The projection structure shows stain deficient bridges between adjacent ribbons but a strong interaction occurs initially in the crystallization process between $\alpha\beta$-units. The analysis of the two-rowed arrays indicate that $(\alpha\beta)_2$ -units of Na,K-ATPase are the initial building blocks of p21 crystals. However, multiple-rowed arrays are also observed consisting of a two-rowed ribbon and one or more adjacent single-rowed arrays. Thus, the membrane fragments may also contain free $(\alpha\beta)$-protomers of the enzyme (Söderholm et al., 1986).

3-D STRUCTURE OF Na,K-ATPase

The three-dimensional structure of Na,K-ATPase as determined by electron microscopy was first obtained from the dimeric membrane crystals (Hebert et al., 1985a). For this purpose we used membrane crystals formed after incubation with vanadate and magnesium and negatively stained with uranyl acetate. The membranes were tilted through a range of angles $\pm$ 60° and Fourier transforms calculated for each projection. The procedure for 3-D reconstruction was essentially that of Henderson and Unwin (1975; Amos et al., 1982). The images of the tilted molecule were synthesized into a three-dimensional model using the "EM" system (Hegerl and Altbauer, 1982) implemented on a NORD-500 computer. The calculated 3-D structure was obtained as sections parallel to the membrane at 5-Å intervals through the unit cell (Fig. 5). The second positive contour line of these

sections were used to construct a balsa tree model of the molecule (Figs. 6-9).

The model shows two symmetrically related rod-like protein regions in the unit cell. The rods are slightly flattened and inclined relative to the c-axis, which runs perpendicular to the membrane. Stain deficient regions are present between adjacent rods and form contact regions between the two rods in a pair as well as between adjacent units in adjacent pairs along the b-axis. The bridge between protein units within a unit cell is located on one side of rods (Fig. 5B) while the bridges which are present on the opposite side are between rods in neighbouring unit cells and oriented in the direction of the b-axis of the crystal (Fig. 5A).

The minimum length of the protein units perpendicular to the membrane is about 100 Å. The contrast variation curve shows a minimum located between -10 and +30 Å, when the midpoint of the rods in the direction of the c-axis is set at 0 Å. This minimum may correspond to the location of the lipid bilayer since the negative stain probably does not penetrate the bilayer (Deatherage et al., 1982). Therefore the Na,K-ATPase protein is asymmetrically distributed in the membrane in relation to the lipid bilayer. It protrudes at least 40 Å on one side of the bilayer which probably corresponds to the cytoplasmic side since labelling studies indicate that much more of the enzyme protein protrudes above the membrane plane on the cytoplasmic than on the extracellular side of the membrane (O'Connell, 1982). Furthermore it can be concluded that the protein projecting on the cytoplasmic side, including the contact region between the protomers, belong to the α-subunit, since very little of the protein of the β-subunit is present on the cytoplasmic side (Ovchinnikov et al., 1987). On the opposite side, which probably corresponds to the extracellular side, the stain-excluding portions protrude about 20 Å and represent in part the β-subunit.

The volume of the unit which contains one of the two rod-shaped structures is 475,000 $Å^3$. This is slightly smaller than the value for cytochrome oxidase crystals (Deatherage et al., 1982) which contains one monomeric protein unit of 146 kD. Therefore it is likely that one rod-shaped unit of the Na,K-ATPase crystal corresponds to one αβ-unit of the enzyme since the widely accepted molecular mass of one αβ-protomer is about 150 kD. Assuming the above location of the lipid bilayer about 42% of the volume of the model is associated with the lipid bilayer, 40% on the cytoplasmic side and 18% on the extracellular surface.

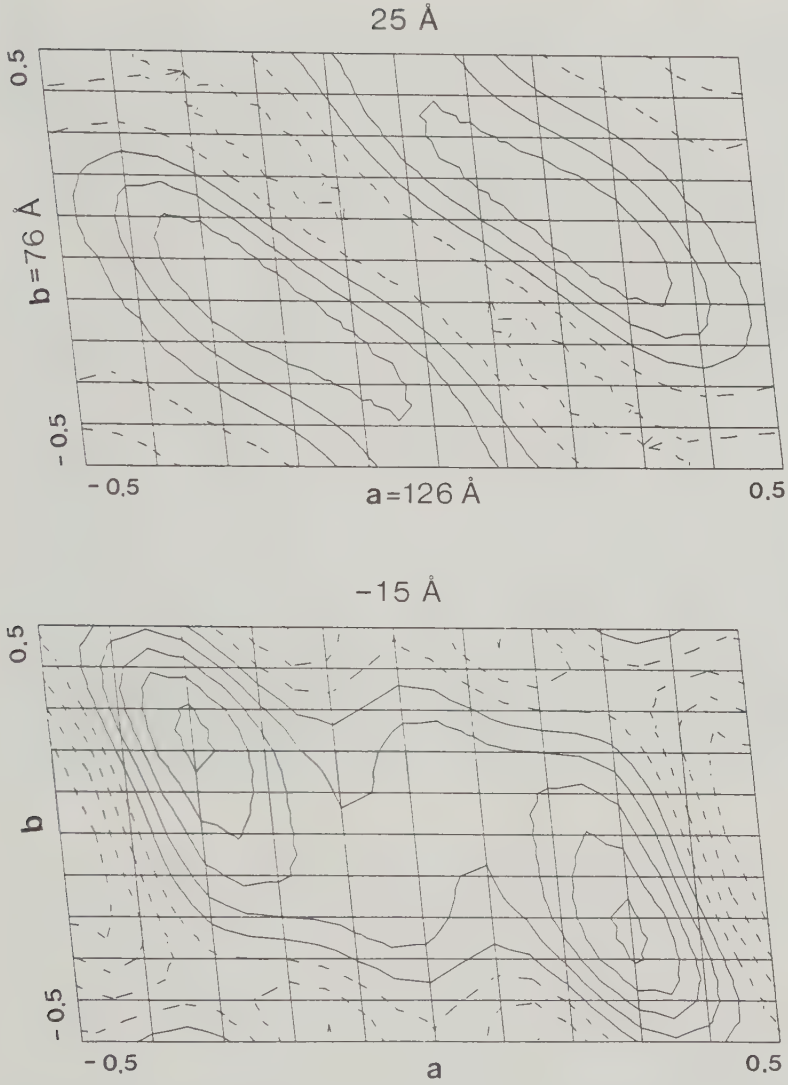

Fig. 5. Sections through the 3-D map of the Na,K-ATPase dimer oriented parallel to the membrane and located +25 and -15 Å from the center of the molecule. The upper section is located close to the extracellular surface of the lipid bilayer while the lower section is a cross-section of the dimer within the cytoplasm but close to the lipid bilayer (from Hebert et al., 1985a).

Ovchinnikov et al. (1985) also determined the three-dimensional structure of the Na,K-ATPase from dimeric crystals of type II and found a similar shape as that described above, except that the contact region between the two protomers was located approximately between the middle parts rather than between the cytoplasmic regions as in our model. Both in the model by Hebert et al. (1985a) and Ovchinnikov et al. (1985) parts of the enzyme protein project above the extracellular part of the lipid bilayer. On the contrary Mohraz et al. (1987) recently reconstructed Na,K-ATPase from dimeric crystals type I and observed no protein projecting above the membrane plane on the extracellular side. In addition the protomers showed different orientations. Nevertheless the model supports the conclusion that the contact region between the protomers belongs to the α-subunit.

COMPARISON WITH OTHER ATPases

The vanadate method for Na,K-ATPase has also been applied to other membrane-bound ATPases. Thus, Ca-ATPase from sarcoplasmic reticulum forms crystalline arrays in media containing vanadate (Dux and Martonosi, 1983; Buhle et al., 1983). In apparent contrast to Na,K-ATPase crystallized regions of Ca-ATPase may be present in untreated, intact cells (Castellani and Hardwicke, 1983). Three-dimensional reconstructions of the vanadate-induced two-dimensional crystals show that the Ca-ATPase molecule in dimeric crystals projects above the lipid bilayer into the cytoplasm in much the same way as in Na,K-ATPase crystals (Castellani et al., 1985; Taylor et al., 1986). Also in Ca-ATPase crystals there is a contact region between protomers located within the cytoplasm and above the surface of the membrane bilayer. However, in contrast to Na,K-ATPase membranes the opposite side of the membrane is featureless in Ca-ATPase crystals. This difference between Na,K-ATPase and Ca-ATPase membrane crystals is consistent with the absence of a β-subunit in Ca-ATPase and the assumption that the major part of the enzyme protein, which projects into the extracellular side of Na,K-ATPase membranes, represent a β-subunit. The similarity between the cytoplasmic projections of the two transport enzymes is also consistent with the conclusion that these parts of the molecules represent α-subunits. Also H,K-ATPase purified from the gastric mucosa can be crystallized with vanadate and projection maps revealed that the enzyme protein form dimeric arrays (Rabon et al., 1986). At the present time three-dimensional reconstructions of this enzyme have not been carried out.

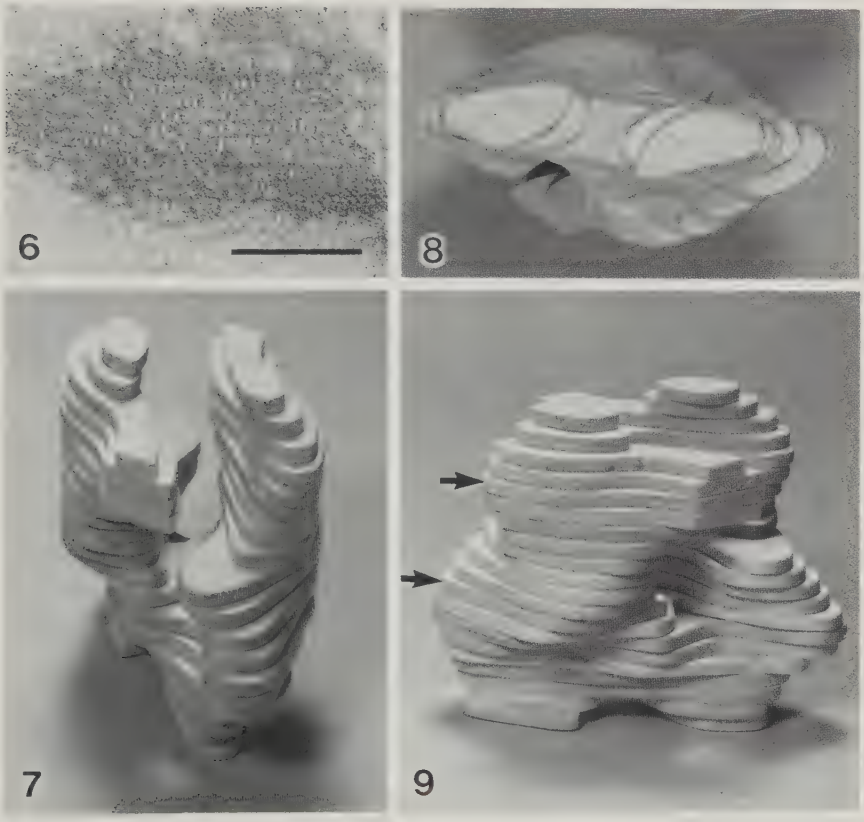

Fig. 6. Electron micrograph of dimeric membrane crystal of Na,K-ATPase used for reconstruction of the three-dimensional structure of the enzyme protein. This image was recorded at a tilt angle of +6°. Bar: 1000 Å.

Fig. 7. Three-dimensional model of Na,K-ATPase. The unit cell contains two flattened protein regions (protomers) related by a dyad axis and with a cleft between the symmetry-related units.

Fig. 8. Three-dimensional model of Na,K-ATPase as observed in the direction of the c-axis and from the cytoplasmic side of the membrane where the two protomeric protein units are in contact.

Fig. 9. The model as observed in a direction about half way between the a and b axes and slightly from above the membrane plane. The lipid bilayer is assumed to be located approximately between the levels indicated by arrows i.e. in the direction of the extracellular side of the molecule (Figs. 6-9 from Maunsbach et al., 1986).

FUTURE PERSPECTIVES

The three-dimensional reconstructions of Na,K-ATPase determined by electron microscopy of protomeric or dimeric membrane crystals demonstrate the shapes of the protein units at a resolution level of about 20-25 Å. At this resolution only some of the main structural characteristics of the molecule can be expected to be resolved. In order to identify possible ion channels or to detect small conformational changes a much higher resolution would be necessary. Presently a resolution of 3.5 Å has been obtained in the projection structure of the purple membrane from Halobacterium halobium (Henderson et al., 1986). These natural membrane crystals show a very high degree of order and much further work will be required in order to obtain large membrane crystals of Na,K-ATPase with a similar degree of order.

As discussed above only a few portions of the three-dimensional model of Na,K-ATPase have been tentatively identified as representing either parts of the α or the β-subunits of the enzyme. Future work will be directed towards identifying specific portions of the enzyme either with lectins, antibodies or other site specific probes, such as exemplified by the identification of the ATPase-site of myosin (Tokunaga et al., 1987). A long term goal will be to determine how the three-dimensional structure of the enzyme relates to the primary structure of the α-subunit (Kawakami et al., 1985; Shull et al., 1985) and β-subunit (Kawakami et al., 1986; Noguchi et al., 1986; Shull et al., 1986).

The Na,K-ATPase crystals which have been analyzed so far by image processing and reconstruction methods have all been induced with vanadate, or other ions, which favour the protein in the E_2-conformation (Cantley et al., 1978; Jørgensen and Karlish, 1980). Two-dimensional crystals have also been induced by ions which favour the E_1-conformation of the enzyme (Skriver et al., 1985) but such crystals have not yet been analyzed in detail by image processing since they tend to be small and less regular than crystals induced in the E_2-conformation. However, if improved E_1-crystals can be obtained and analyzed by electron microscopy at very high resolution it may be possible to obtain information regarding the changes in shape of the enzyme protein which are associated with its transport cycle.

An alternative approach to the analysis of the structure of Na,K-ATPase is to try to grow three-dimensional crystals of the enzyme suitable for X-ray crystallographic structure determination. A protein complex from the photosynthetic reaction center of the purple bacterium Rhodopseudomonas viridis has been

crystallized by salt precipitation in the presence of detergent and a small amphiphile (Michel, 1982). This is the first protein from a biological membrane for which the structure has been determined at atomic resolution (Deisenhofer et al., 1985). Three-dimensional crystals of Na,K-ATPase have not yet been obtained. However, stacking of crystallized Ca-ATPase membranes have been observed (Dux et al., 1987) suggesting that also the transport ATPases may eventually be crystallized in three dimensions.

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SEQUENCE ANALYSIS OF EXPOSED DOMAINS OF MEMBRANE-BOUND Na^+,K^+-ATPase. MODEL OF TRANSMEMBRANE ARRANGEMENT

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Determination of complete primary structure of Na^+,K^+-transporting adenosine triphosphatases from diverse sources (Ovchinnikov et al.,1985,1986a,b; Kawakami et al., 1985; Nogushi et al.,1986; Shull et al.,1985,1986) initiated a new stage in studying the enzyme molecular organisation - a direct positioning of certain structural elements of subunit polypeptide chains. The urgent task here is identification of exposed and intramembrane domains, i.e. design of a two-dimensional scheme for the transmembrane protein folding.

Calculation of the hydrophobicity profile of the α-subunit allowed prediction of eleven probable transmembrane clusters (Ovchinnikov et al.,1986b): 89-114,123-142,284-306, 313-341,530-554,569-597,780-803,842-867,909-930,946-971,973-994. A methodological approach for modelling the enzyme molecule in the membrane is based on limited proteolysis of the membrane-bound Na^+,K^+-ATPase. It implies only the exposed protein regions to be proteolytically attacked and intramembrane moiety to be safely protected by the lipid environment. Earlier we found conditions for trypsinolysis of the membrane-bound enzyme (0.1 N NH_4HCO_3, pH 7.3, 1% trypsin, 10 min) providing exhaustive hydrolysis of the exposed regions of the polypeptide chain of the catalytic subunit (Modyanov et al.,1987). Among the water-soluble peptides isolated from the hydrolysate we identified three fragments 529-544,545-589,590-598 and thus moderately hydrophobic stretches 540-554 and 569-597 could not be considered as embedded into the lipid bilayer. Peptides 913-931 and 973-998 were identified through directed isolation of cysteine-

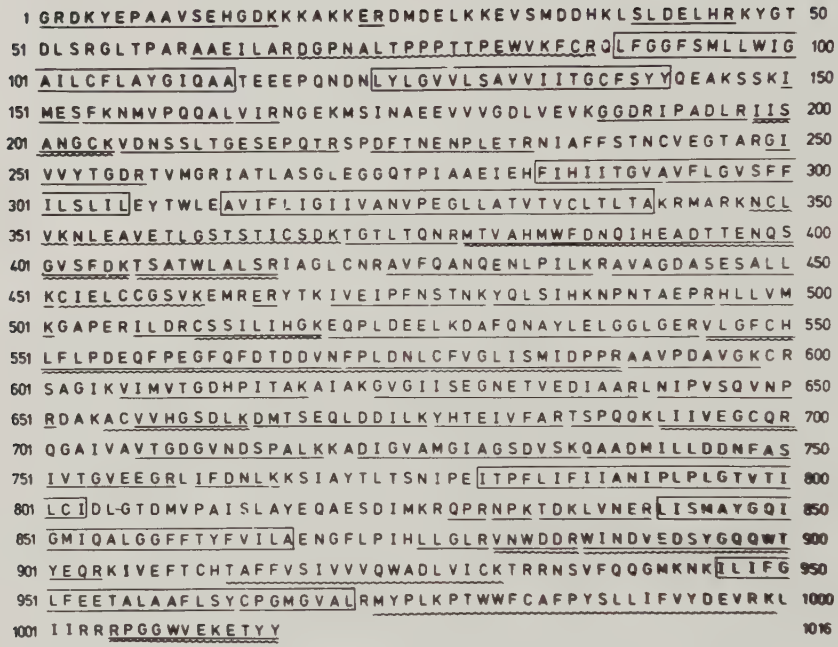

Figure 1. Amino acid sequence of α-subunit. Peptides resulted from limited tryptic hydrolysis of membrane-bound enzyme are underlined: ——— before and ~~~~ after chemical modifications of tryptophan residues. Suggested transmembrane segments are framed.

and tryptophan-containing peptides from the water-soluble hydrolysate fraction including conversion of tryptophan residues into SH-tryptophan and selective covalent chromatography on Thiol-Sepharose (Ovchinnikov et al.,1987a). It means that rather hydrophobic sequences 909-930 and 973-994 are in fact exposed outside the membrane. Sequences of isolated peptides (in total about 600 amino acid residues) cover the following regions of the polypeptide chain: 1-87, 150-257,348-766,826-841, and 973-1016 (Fig.1), thus providing experimental evidence of the exposure of these regions in the α-subunit. Other regions of the polypeptide chain can be considered as a sum of membrane-bound fragments. Of

eleven probable transmembrane segments previously suggested only seven belong to this portion of the molecule. According to the hydrophobicity profile their coordinates are: 89-114,123-142,284-306,313-341,780-803,842-867,946-971. More precise determination of boundaries of intra- and extramembrane regions is limited because of trypsin specifity. In addition, proteolysis may induce the slight disturbance in the spatial disposition of the protein fragments originally located in the polar membrane zone.

Cytoplasmic location of hydrophilic regions 1-87, 143-283 and 342-779 situated ahead of rod I and between II-III and IV-V membrane rods seems obvious, since they include the known markers of the intracellular region of the α-subunit: components of the catalytic site and the points of primary proteolytic cleavage characteristic of E_1 and E_2 enzyme forms. The orientation of the hydrophilic loops 868-945 and 804-841 relative to the plasma membrane was proved by means of monoclonal antibodies IIC_9 and VG_2 interacting with smears or viable cells of porcine kidney embryonic (PKE) cell line upon immunofluorescent analysis, respectively (Modyanov et al.,1987). Their antigenic determinants are located in peptides 887-904 (IIC_9) and 810-825 (VG_2).

To position the C-terminus of the α-subunit, antibodies to the synthetic peptide corresponding to fragment 999-1008 of the polypeptide chain (p999) were raised in rabbit and affinity-purified on immobilized p999. In solid phase ELIZA these antibodies (α-p999) linked membrane-bound ATPase. The blot from SDS-PAGE-resolved microsomes of PKE stained with α-p999 antibodies showed only one band with mobility corresponding to ATPase α-subunit. When viable PKE cells were stained with α-p999 antibodies and fluorescein-labeled antibodies to rabbit IgG, all cells had fluorescence 3-4-fold higher as compared to the control stained with normal IgG (analysis with Epics-V fluorescence-activated cell sorter). Trypsin treatment of PKE cells reduced considerably the density of the p999 epitope on the cell surface as judged by the Epics-V analysis. Thus C-terminus of the α-subunit is located outside the cell (Ovchinnikov et al., 1987b). In addition to the exposed C-terminus and hydrophilic loop 804-841 the extramembrane region of the molecule involves short junctions I-II and III-IV of membrane segments (115-122 and 307-312).

The first stage of the tryptic hydrolysis of the membrane-bound enzyme yielded the preparation containing intact glycoprotein serving as suitable model to study the spatial arrangement of the β-subunit. To overcome its resistance to proteolysis, the original preparation was preliminary treated with neuroaminidase to remove the shielding effect produced by the carbohydrate chains evenly distributed on the long C-terminal part or with β-mercaptoethanol for reduction one or two internal disulfide bonds (Ovchinnikov et al.,1987a). Our results show the latter facilitates fast exhaustive tryptic hydrolysis of the exposed domains of the β-subunit. Thus glycoprotein stability is possibly determined by disulfide bridges. Special attention was paid to identification of the fragments that could be ascribed to the membrane sector due to their high hydrophobicity index. The polypeptide chain of the β-subunit can involve up to four (23-67,197-214,223-260) intramembrane rods (Ovchinnikov et al.,1986b). However, the experimental results show that peptides 22-26,71-84,182-202, 223-247, and 248-272 belong to the exposed protein areas (Fig.2). So the limited proteolysis unraveled only one hydrophobic region (34-61) in the membrane-bound β-subunit. This fragment may span the lipid bilayer once. The C-terminal part of the protein molecule (62-302) forms the single extracellular domain, and the N-terminal hydrophilic region (1-33) is, obviously, exposed to the cytoplasm.

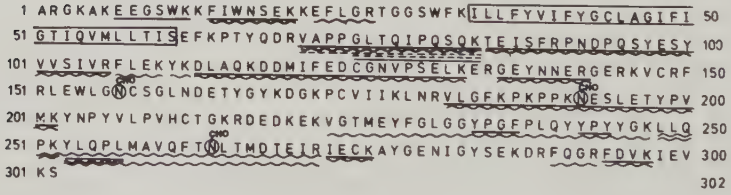

Figure 2. Amino acid sequence of β-subunit. Peptides yielded from tryptic hydrolysis of ----- membrane-bound β-subunit (5%, 60 min), ——— desialilated membrane-bound β-subunit (2.5%, 180 min), ~~~~~ reduced membrane-bound β-subunit (5%, 20 min). Proposed transmembrane segment is framed.

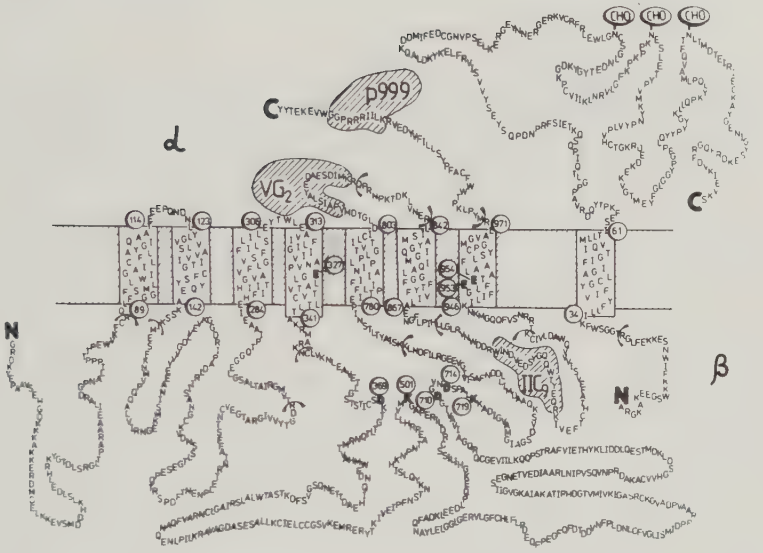

Figure 3. Scheme for spatial organisation of αβ-protomer of Na^+,K^+-ATPase. Hatched regions of α-subunit polypeptide chain correspond to epitopes of monoclonal antibodies IIC_9, VG_2 and p999. The areas of disposition of isolated extramembrane peptides are bracketed.

The above structural analysis became an experimental basis for the detailed model of the transmembrane organisation of the enzyme αβ-protomer (Fig.3) (Ovchinnikov et al., 1987a). It allowed determination of the composition of the Na^+,K^+-ATPase membrane sector that is actually involved in cation transfer across the hydrophobic barrier of the lipid bilayer. Of eight transmembrane segments having 20-28 residues, only the 4th and 7th rods of the α-subunit include three charged residues of glutamic acid (327,953,954) located in the hydrophobic portion of the lipid bilayer. This unique situation implies that carboxyl groups could directly participate in the transport. Thus the primary targets for site directed mutagenesis, that would throw light on the functional role of certain amino acid residues in the vector

TABLE 1. Comparison the secondary structures in the native Na^+,K^+-ATPase and the membrane-bound products of its two-stage trypsinolysis.

Preparation/Type of structure	Raman data				Predictional techniques		
	α - HELIX (%)		β - STRUCTURE (%)	UNDEFINED CONFORMATION (%)	α-HELIX (%)	β-STRUCTURE (%)	UNDEFINED (%)
	ORDERED	DISORDERED					
INTACT Na^+,K^+-ATPase	42 ± 4 (554 ± 52)		30 ± 4 (395 ± 52)	28 ± 4 (369 ± 52)	39	22	39
	28 ± 4 (369 ± 52)	14 ± 4 (185 ± 52)					
α-SUBUNIT HYDROPHOBIC DOMAIN AND THE INTACT β-SUBUNIT	48 ± 4 (282 ± 24)		32 ± 4 (188 ± 24)	20 ± 4 (117 ± 24)	42	22	36
	15 ± 4 (88 ± 24)	33 ± 4 (193 ± 24)					
HYDROPHOBIC DOMAINES OF α- AND β-SUBUNITS	78 ± 4 (230 ± 12)		0	30 ± 4 (99 ± 12)	64	4	28
	24 ± 4 (79 ± 12)	46 ± 4 (151 ± 12)					

transfer of cations, are evident.

Raman spectra of active Na^+,K^+-ATPase and membrane-bound products of its stepwise trypsinolysis were registered to calculate the secondary structure of hydrophilic and hydrophobic regions for α- and β-subunits. Consequent comparison these results for the native enzyme and its membrane-bound preparations including α-subunit hydrophobic regions as well as the intact β-subunit or only hydrophobic regions of the both subunits demonstrated unambiguous that (i) membrane-bound hydrophobic parts of polypeptide chains of Na^+,K^+-ATPase subunits (230 amino acid residues) are in the α-helical conformation; (ii) the α-helix (up to 270 residues) and β-sheet (up to 200 residues) are estimated to form hydrophilic (mainly cytoplasmic) domain of the Na^+,K^+-ATPase α-subunit; (iii) the exoplasmic hydrophilic domain of the β-subunit is shown to include several antiparallel β-pleated sheets (up to 180 residues) and the small amount of α-helix and undefined conformations (Table 1). The model of the secondary structure organisation for the hydrophilic domains as well as eight hydrophobic transmembrane segments of the Na^+,K^+-ATPase membrane-bound complex

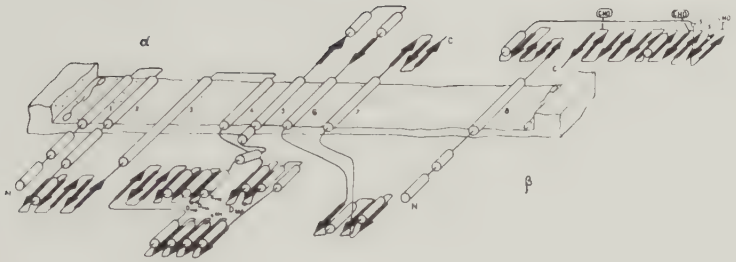

Figure 4. Structural diagram of the Na^+,K^+-ATPase molecule based on Raman data as well as predicted domain structure and hydrophobicity plots. α-Helical segments are marked with cylinders. Arrows indicate β-pleated sheets.

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was obtained from Raman data and statistic predictional techniques (Fig.4).

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SPATIAL STRUCTURE AND MEMBRANE ORGANIZATION OF PIG KIDNEY Na,K-ATPase

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Modern electron microscopy is a useful approach to the investigation of membrane proteins. In spite of the relatively low resolution valuable structural information can be obtained. Negative staining of the membrane fragments containing Na,K-ATPase as the only protein revealed the enzyme as globular particles randomly distributed on the membrane surface. Slow cooling (0.1 °C/hr) of the membrane suspension from 37° to 2-4 °C in Mg^{2+}- or PO_4^{3-}-containing solutions leads to particle aggregation; sometimes as a result of this aggregation membraneous ATPase gives regular 2D-crystals (Fig. 1). Depending on the conditions Na,K-ATPase crystallizes in three different forms (Hebert et al., 1982; Demin et al., 1984). One of them is best suited for three-dimensional data collection since it dominates in the presence of Mg^{2+}, VO_3^- and K^+ in the crystallization medium. Fig. 2 presents the projected map of these crystals. The unit cell of the crystals has the following parameters: a = 66 Å, b = 118 Å and γ = 108°, and contains two protein density maxima. Diffraction maximum phases are approximately 0° or 180° (standard deviation at phase center refinement does not exceed 17°) and the filtered crystal images clearly indicate the presence of a two-fold rotation axis normal to the membrane plane. It means that the crystals belong to the p21 two-sided plane group (Holser, 1958).

Three-dimensional reconstruction was calculated in space group P2 on the basis of data from five independent tilt series. The spatial structure obtained in this way of the crystal unit cell is presented in Fig. 3A as cross-sections 7.5 Å thick parallel to the membrane plane. The unit cell consists of two identical stain excluding regions. Its calculated volume is 340,000 $Å^3$. If we

Fig. 1. Electron micrograph of negatively stained 2D-crystal of the Na,K-ATPase.

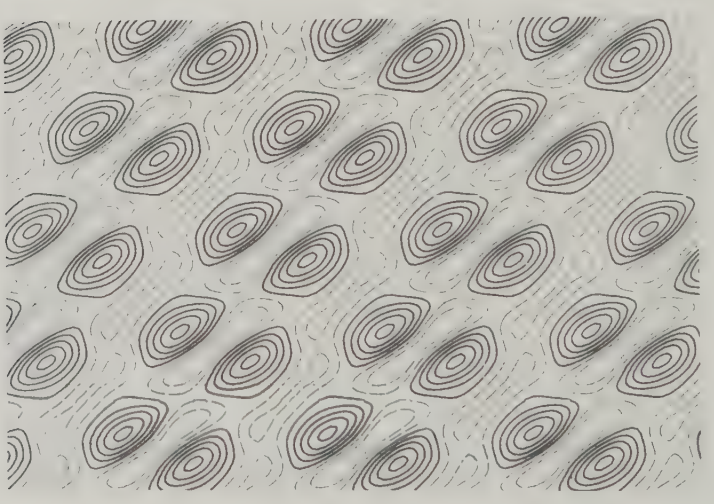

Fig. 2. Computer reconstructed image of the Na,K-ATPase crystal. Stain excluding regions are drawn with unbroken contour lines, while negative stain regions have dashed lines.

assume a partial protein volume of 1.3 $Å^3/D$ (Henderson and Unwin, 1975) its mass will be 260 kD that is in a good agreement with the molecular weight of two $\alpha\beta$ -protomers (280 kD). As the protein is crystallized without membrane solubilization, the protomers have identical orientation in regard to the membrane plane. In the unit cell protomers are linked by the two-fold rotation axis and have a contact area approximately in their middle part.

The protomer length is about 100 Å, which exceeds the normal thickness of the lipid bilayer. Apparently considerable parts of the protein are exposed on the membrane surfaces. The hydrophobicity profile of the Na,K-ATPase amino acid sequence (Ovchinnikov et al., 1986a) together with data on hydrophilic and hydrophobic labeling of the protein in open membrane fragments and "inside-out" proteoliposomes indicated that the membrane-embedded part of the protein consists of not more than 25% of the mass for each subunit, its total mass not exceeding 40 kD. Thus, 75% of the subunit protein mass is distributed between cytoplasmic and extracellular domains of the protomer. Fluorescamine labeling showed that for the α-subunit the cytoplasmic domain is about 3 times as large as its extracellular region (Ovchinnikov et al., 1985). The hydrophilic part of the β-subunit is exposed only on the outer surface of the membrane (Dzhandzhugazyan et al., 1981b; Ovchinnikov et al., 1985). Accordingly, peripheral domains of the protomer on both sides of the membrane should be of about 50 kD.

Since the total volume of the protomer (170,000 $Å^3$) corresponds to 150 kD such hydrophilic domains occupy 4 cross-sections from one side and 4 from the opposite (model in Fig. 3A). The 5 sections in-between form the hydrophobic segment; its height of 40 Å being in good agreement with the conventional thickness of the lipid bilayer.

To elucidate the subunit topography of protomers we investigated the structure of the β-subunit which can be isolated separately (Dzhandzhugazyan et al., 1981a). As in case of Na,K-ATPase, the β-subunit preparation consists of membrane fragments containing the β-subunit as the only protein. Slow cooling of the membrane suspension to 4 °C in the presence of Mg^{2+} or Ca^{2+} results in 2D-crystal formation (Ovchinnikov et al., 1986b). Filtered images of the crystals are presented in Fig. 4. Unlike Na,K-ATPase, the β-subunit crystallizes in the absence of VO_3^- and the obtained crystals are thermally quite stable. Since crystallization of the enzyme was carried out in the

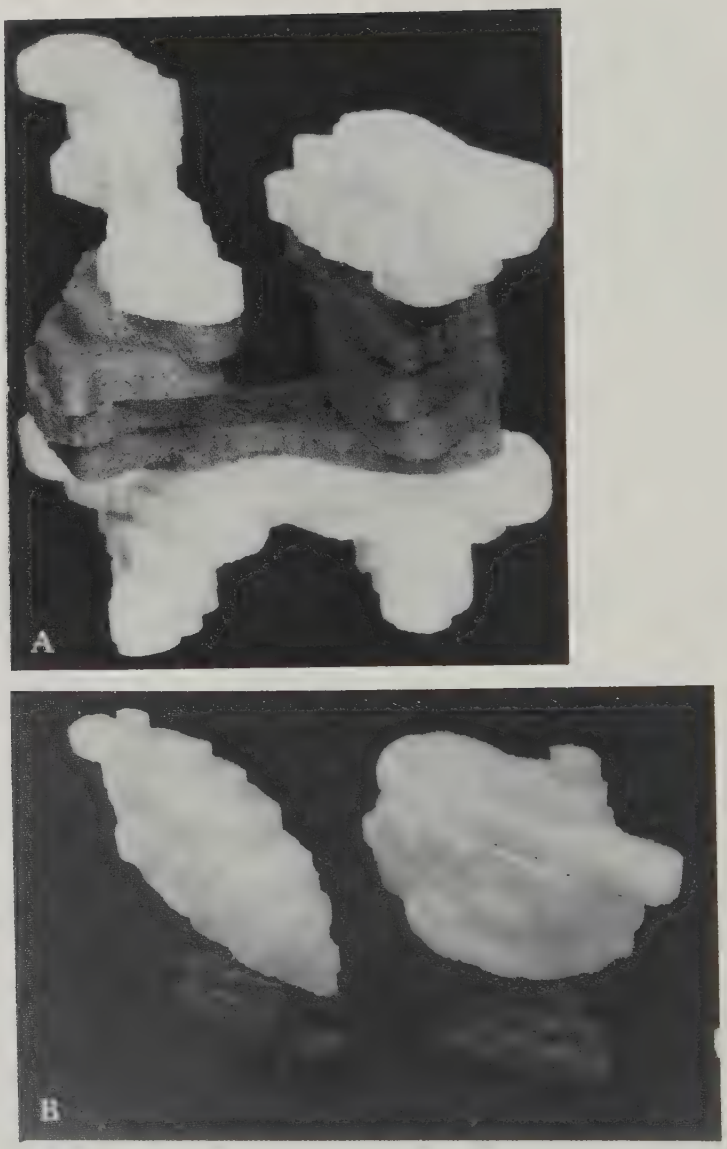

Fig. 3. 3D-models of the Na,K-ATPase (A) and β-subunit (B) crystal unit cells. Intramembrane portions of the proteins are dark.

presence of Mg^{2+}, we investigated the β-subunit crystals obtained in the presence of the same ion. The unit cell of the crystals had the following parameters: a = 77 Å, b = 177 Å, γ = 63°. Crystals pertain to the p21 two-sided plane group because the phases of diffraction maxima are about 0° or 180° and the filtered images clearly demonstrate a 2-fold rotation axis normal to the membrane plane (Holser, 1958).

The unit cell contains two main stain excluding regions and an additional density region looks like a strip (Fig. 4, a and b, respectively).

3D-structure calculation was made in space group P2 using the electron micrographs in the ±60° tilting range. A 3D-model of the two main density regions of the unit cell is shown in Fig. 3B as sections 6.5 Å thick parallel to the membrane plane. The calculated molecular mass of each region is about 40 kD, which perfectly agrees with the molecular weight of the β-subunit (35 kD). Apparently an additional stain excluding region (b in Fig. 4B) represents the rest of the lipid bilayer. This suggestion could be confirmed by its vertical dimension (about 40 Å) and location along the c-axis of the unit cell (see below).

The spatial structure of the β-subunit is characterized by marked asymmetry of mass distribution along the axis perpendicular to the membrane plane. Immunochemical and chemical modification data indicate that hydrophilic parts of the β-subunit polypeptide chain form one domain exposed on the extracellular membrane surface (Dzhandzhugazuan et al., 1981b; Ovchinnikov et al., 1985). The hydrophobicity profile of the β-subunit amino acid sequence evidently indicates no more than four transmembrane stretches (Ovchinnikov et al., 1986a). Thus, the molecular mass of the hydrophilic domain is about 25 kD. This coincides with the data obtained from chemical modification of the protein with hydrophilic reagents (Dzhandzhugazyan and Jørgensen, 1985). Accordingly, in Fig. 3B the hydrophilic domain is represented by 8 sections in the wide part of the model, while its narrow part (6 sections) represents the intramembraneous domain. The height of the membrane portion is about 40 Å, in accordance with the normal thickness of the lipid bilayer. It is notable that the additional stain excluding region (b in Fig. 4) is disposed in the same region of 3D-crystals.

The characteristic feature of β-subunit crystals is the absence of any significant contacts between the adjacent polypeptides in the unit cell. αβ-protomers of Na,K-ATPase crystals

Fig. 4. Computer reconstructed images of the ß-subunit 2D-crystals obtained in the presence of Ca^{2+} (A) and Mg^{2+} (B). Only stain excluding regions are drawn.

have a contact site which is asymmetrically positioned relative to the membrane (Fig. 3A). It is evident that Na,K-ATPase dimers are formed by interactions of α-subunits, and contact sites should be located on the intracellular membrane surface, where the larger part of the α-subunit is exposed (lower part of the model in Fig. 3A). Consequently, in the Na,K-ATPase dimer the hydrophilic domains of β-subunits are disposed in the extracellular parts of the protomers, far from the contacting site formed by two α-subunits.

Crystallization and negative staining can drastically change the supramolecular organization of proteins. Due to the extremely rapid sample cooling, freeze-fracture electron microscopy can be used for visualization of the "native" organization of the membane proteins. This technique reveals Na,K-ATPase as globular complexes of uniform size: they are 95-105 Å in diameter (Demin et al., 1981). Such complexes can be seen not only in plasma membrane fragments but also in proteoliposomes prepared by functional reconstitution of the detergent solubilized enzyme. Consequently packing of the enzyme subunits in complexes of this size is a characteristic feature of the Na,K-ATPase molecule organization. On the basis of quantitative analysis of freeze-fracture micrographs, the protein molecular weight of such complexes is calculated. The main principle of the calculation is shown by the equation:

$$M.W. = \frac{\text{Protein mass in 1 ml} \cdot \text{Avogadro's number}}{\text{Number of the complexes in 1 ml}}$$

Protein mass should be measured by an independent method. The number of protein complexes in the volume unit can be determined by the morphometrical analysis of the micrographs (Demin et al., 1981). Investigation of a number Na,K-ATPase samples of different protein concentrations revealed the molecular mass of a single complex to be 500-600 kD. As the molecular weight of the $\alpha\beta$ -protomers is about 150 kD, one can conclude that the structural complex consists of 4α- and 4β-subunits.

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THE THIRD TYPE OF ALPHA-SUBUNIT OF Na,K-ATPase

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ABSTRACT

The cDNA encoding the third type of alpha-subunit of NaK-ATPase was found in rat brain cDNA library. The deduced amino acid sequence was very similar to those of alpha- and alpha plus-subunits. The third one was suggested to be highly ouabain-sensitive from its aminoacid sequence. From Northern blot analysis the third type of the subunit was not expressed in adult rat kidney and heart.

INTRODUCTION

Two forms of the NaK-ATPase were reported in mammals (Urayama and Nakao, 1979). One, called the kidney-type NaK-ATPase, was found in most organs and the other, called the brain-type Na,K-ATPase exists in axolemma (Sweadner, 1979) and adipocyte plasma membrane (Lytton et al., 1985). The two rat isozymes differ in their affinity for ouabain (Urayama and Nakao, 1979) and in the molecular mass of the alpha-subunit, and have distinct antigenic determinants (Sweadner and Gilkeson, 1985). The alpha-subunit of the brain-type enzyme, designated as alpha plus, exhibits larger molecular weight than the alpha-subunit of the kidney type, greater by 5-8 kDa as estimated from SDS-PAGE analysis. Very recently the existence of the third type of alpha-subunit in rat brain (alpha III) was suggested from cDNA cloning (Shull et

al., 1986; Hara et al., 1987). In this paper we present the cloning of a cDNA encoding alpha III and comparison of the aminoacid sequence of the alpha III subunit with the rat alpha- and other mammalian alpha-subunits. Ouabain sensitivity of the new subunit is discussed.

RESULTS AND DISCUSSION

Isolation of cDNA Clones

The Okayama-Berg cDNA library (Okayama and Berg, 1983) constructed from rat brain mRNA was screened by hybridization using a fragment of cDNA encoding the alpha-subunit of human NaK-ATPase as a probe. Fifteen positive clones that hybridized with the probe were obtained from around 50 000 transformants. These clones could be divided into two groups (group A, 11 clones; group B, 4 clones), from the restriction profiles with Hinf I or Bam HI. The clones harboring the longest insert cDNA in group A was found to encode rat alpha-subunit of NaK-ATPase from DNA sequence analysis. In order to obtain a clone carrying a longer insert in group B, two further screenings were carried out using the same cDNA library. The second screening yielded 9 positive clones out of 200 000 transformants. The third screening was done using a 5'-side cDNA fragment of the longest insert obtained from the second screening. Out of 1 000 000 colonies, 26 positive clones were obtained in the third screening. The resultant clone carrying the longest insert (3.5 kbp), pRNKA131, was subjected to DNA sequence analysis.

Analysis of cDNA Clones

The cDNA insert of clone pRNKA131 consisted of 3039 bp of coding region, 140 bp of 5'-noncoding and 378 bp of 3'-noncoding regions. It showed 72% homology in nucleotide sequence with the cDNA coding rat alpha-subunit and the deduced amino acid sequence exhibited 85% homology with that of the alpha-subunit of rat kidney-type NaK-ATPase. The reported N-terminal amino acid sequence of the rat alpha plus-subunit (Lytton, 1985) was not found in our sequence. Therefore it is likely that the cDNA insert of pRNKA131 encodes other kind of alpha-subunit of Na,K-ATPase. We suspect that the cDNA of pRNKA131 might encode a third type of alpha-subunit of the rat brain Na,K-ATPase because at

least three types of alpha-subunit were suggested to exist in chicken brain (Takeyasu et al., 1986).

Comparison of the Amino Acid Sequences of Na,K-ATPase from Various Sources

The degree of homology in amino acid sequence between the rat alpha III and rat alpha plus, rat, human, sheep, pig or Torpedo alpha-subunits was no less than 84% and especially high similarity was found at the membrane spanning domains (M1-M6). The six proteins were virtually the same in terms of amino acid sequence and the locations of the Asp residue phosphorylated during the turnover (Walderhaug et al., 1985), the FITC (Farley et al., 1984), FSBA (Ohta et al., 1986) or ClRATP (Ovchinnikov et al., 1987) binding sites and the membrane spanning domains.

```
                                    120
                                     ▼
                    Torpedo         -V--VDN-A---
highly              Human           ------------
 ouabain            Pig             QAATEEEPQNDN
  sensitive         Sheep           ------------
                    Rat alpha III   --G--DD-SG--
low sensitive ....  Rat alpha       RS------P--D
                        --- M1----              -----M2----
```

Fig. 1. Amino acid sequence between M1 and M2. Residues identical to the pig sequence are represented by -.

Ouabain Sensitivity of Alpha III Subunit

It is known that ouabain inhibit the enzyme from extracellular surface, and it binds to the alpha-subunit of the enzyme. Therefore the ouabain binding site should be located on or near the extracellular surface of the alpha-subunit, taking into account that ouabain cannot easily penetrate the lipid bilayer. It was also reported that the affinity of NaK-ATPase to ouabain varies depending on species and tissues. Thus it is reasonable to suppose that the affinity of the enzyme to ouabain might relate to the structure of the extracellular domain of the enzyme. The extracellularly exposed domains are suggested to be the junctions between M1 and M2, M3 and M4 and M5 and M6. Comparing the amino acid

sequence of ouabain low sensitive rate alpha with those of highly sensitive human, pig, sheep, torpedo alpha subunit, there was found no unique replacement of the amino acid to the rat alpha-subunit in the six membrane spanning domains, in the junction between M3 and M4 or in the junction between M5 and M6. Two other membrane-spanning domains (M7, M8) were proposed (Shull et al., 1985). No replacement was found in the regions corresponding to M7 and M8 or in the junction between M7 and M8. However, a few replacements were found in the junction between M1 and M2 as shown in Fig. 1.

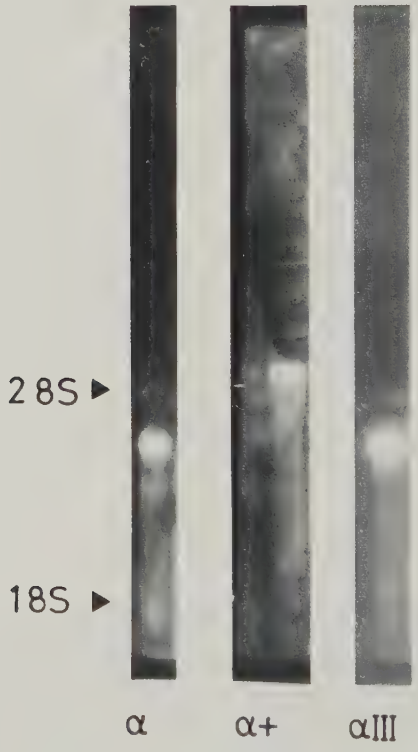

Fig. 2. Northern blot hybridization of total RNA from rat Brain. P-32 labelled 3'untranslated regions of cDNAs encoding rat alpha, alphaplus and alpha III were used as probes.

The amino acid replacements unique to the rat kidney-type enzyme occurred at Arg 113 and Asp 124 as shown in Fig. 1. Replacements at Ser 114 and Pro 121 are not unique to the rat kidney-type because other replacements by other amino acids occur at positions 114 and 121 in Torpedo. Amino acids at these positions may be variable among different species. Residues Gln 113 and/or Asn 124 seemed to be general in the highly ouabain-sensitive Na,K-ATPase alpha-subunit. The replacement of Gln by Arg at position 113 yields an additional positive charge and that of Asn by Asp at position 124 gives a negative charge on the outer domain of the enzyme. A change in the distribution of charge may cause some structural change in the extracellular domain of the enzyme and thus decrease the affinity to ouabain. Taking into account the common structure with highly ouabain-sensitive alpha-subunit discussed above, the NaK-ATPase which contains alpha III-subunit may exhibit high ouabain-sensitivity.

Expression of Alpha-subunit Groups

Northern blot hybridization analysis revealed that alpha, alpha plus and alpha III mRNA existed in brain tissues (Fig. 2) but alpha III mRNA was not detected in adult rat heart and kidney as reported by Young and Lingrel (1987). The alpha III might be specific to central nerves tissues.

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THREE-DIMENSIONAL STRUCTURE OF PROTOMERIC RENAL Na,K-ATPase

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INTRODUCTION

Incubation of purified Na,K-ATPase in membrane-bound form with different ligands under specific conditions induces two-dimensional crystals of the enzyme (Skriver et al., 1981, 1985). Such crystals have been used to determine the projection structure of the enzyme perpendicular to the membrane plane and it has been demonstrated that Na,K-ATPase can form two-dimensional crystals with either one or two molecules in the unit cell (Hebert et al., 1982, 1985a; Mohraz and Smith, 1984; Zampighi et al., 1984; Demin et al., 1984). The three-dimensional structure of the dimeric form of the enzyme has previously been determined with electron microscopy and image processing (Hebert et al., 1985b; Ovchinnikov et al., 1985; Maunsbach et al., 1986; Mohraz et al., 1987). In this paper we present the first three-dimensional reconstruction of the Na,K-ATPase protein in the protomeric crystal form and compare this model with the model obtained for the dimer.

MATERIALS AND METHODS

Na,K-ATPase in membrane-bound form was isolated from the outer medulla of rabbit and pig kidney (Jørgensen, 1974; Skriver and Maunsbach, 1983) and subsequently incubated with 3 mM NH_4VO_3 and 3 mM $MgCl_2$ in 10 mM imidazole buffer, pH 7.0. Negative staining was performed with 1% uranyl acetate and the specimens were examined in a JEOL 100CX electron microscope at a magnification of 54,000X. Tilt series were recorded using 6°

intervals between -60° and +60°. Electron micrographs were digitized into 512 x 512 pixel squares with an increment and a spot size of 25 &m. The "EM" program system (Hegerl and Altbauer, 1982) was used for the image processing performed on NORD-500 and &VAX computers. Correlation averaging (Saxton and Baumeister, 1982) was applied as described earlier for Na,K-ATPase crystals (Hebert et al., 1985a). The relative alignment of the projections of the tilt series, the combination of data into a complete three-dimensional data set and Fourier transformation followed the principles outlined in Amos et al. (1982). A self-consistency criterion for the three-dimensional reconstruction process showed high correlation between the model and the experimental projections.

RESULTS AND DISCUSSION

An electron micrograph of a protomeric two-dimensional membrane crystal of Na,K-ATPase is shown in Fig. 1. Correlation averaging shows that such crystals have a p1 symmetry with one asymmetric unit in the unit cell. On the basis of size it has been concluded that the repeating unit of this type of crystal consists of one §£-protomer of Na,K-ATPase (Hebert et al., 1982). In the present work the three-dimensional structure of the protein unit in a p1 crystal was obtained at a resolution of 25 Å after Fourier transformation of the experimental data based on the tilt series (Figs. 2 and 3). The large central part of the model has an

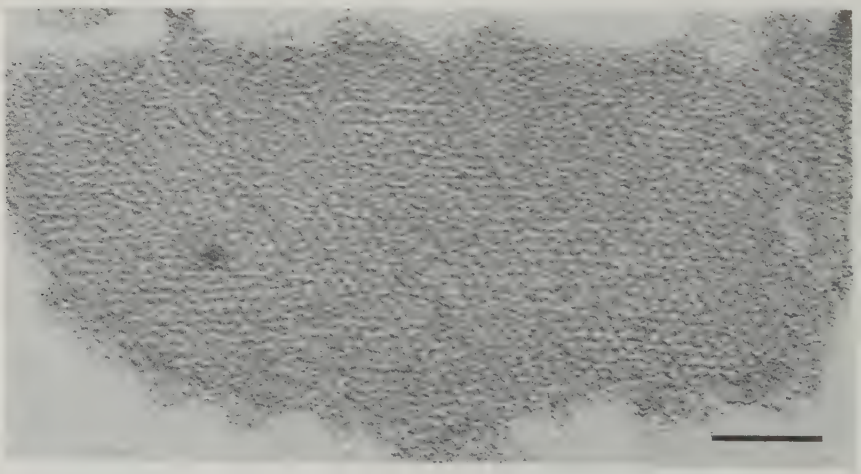

Fig. 1. Electron micrograph of a two-dimensional crystal of renal Na,K-ATPase. Bar: 500 Å.

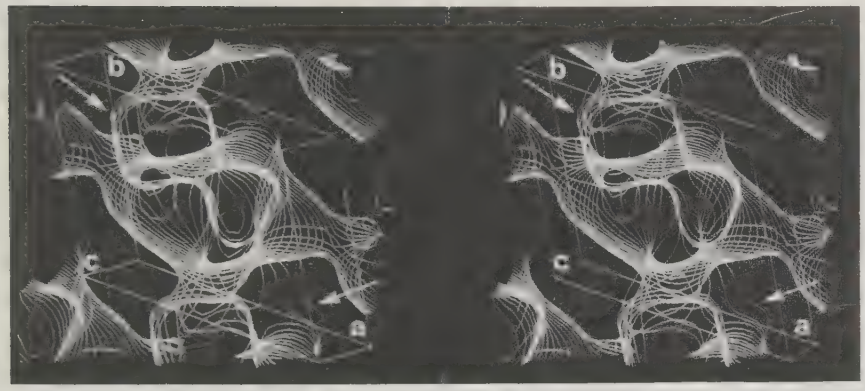

Fig. 2. Stereoscopic drawing of the reconstructed model of Na,K-ATPase in p1 crystal as viewed approximately perpendicular to the plane of the membrane (along the c-axis). The model has been sectioned parallel to the membrane plane and the unit cell has been depicted. The elongated and approximately horizontal central domain is extended along the a-axis of the unit cell. The peripheral domains on both sides of the structure (arrows) are shifted laterally along the b-axis. Scale: 1 mm = 1.8 Å.

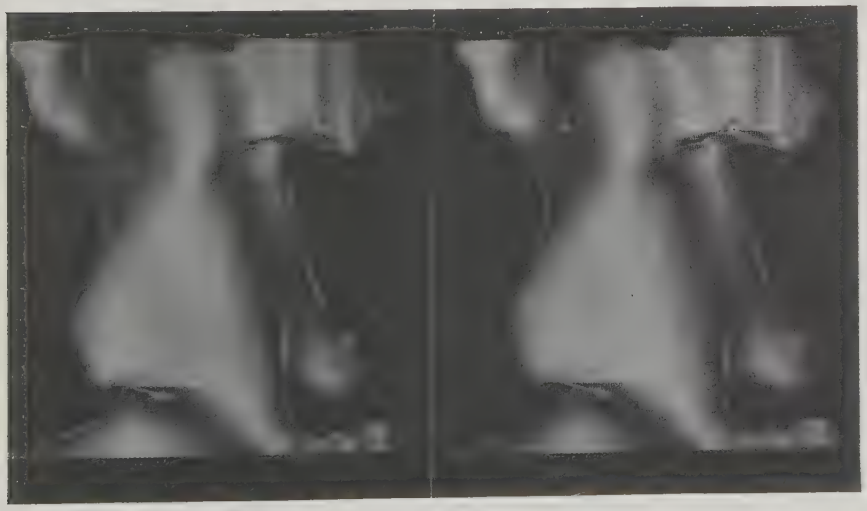

Fig. 3. The three-dimensional protein domain enclosed in the unit cell viewed parallel to the membrane plane. The vertical axis corresponds to the c-axis while the b-axis runs horizontally. Equidistant density sections within a vertical distance of 100 Å was used to produce the computer graphics model.
Scale: 1 mm = 1.6 Å.

ellipsoid shape. The peripheral domains are smaller and laterally translated in relation to the central part. The vertical distance, i.e. the thickness of the model as shown in Figs. 2 and 3 is approximately 100 Å. With the chosen contour cut-off level the volume of the model is 150,000 $Å^3$. Assuming a partial specific volume of 0.725 cm^3/g (Deatherage et al., 1983) the contoured structure corresponds to a protein having a molecular weight of 125,000. Thus, we conclude that the model corresponds to one $\alpha\beta$-unit of the protein.

The structure of the protomeric form of the enzyme was compared with the model of the $(\alpha\beta)_2$-unit obtained from the crystals of the p21 type. This was done by constructing a dimer consisting of two protomeric models related by a two-fold axis. The best fit, as judged by visual comparison of the models presented and interactively rotated on a graphic work station, was obtained when the unit cell axes of the two crystals were approximately parallel. The unit cell dimension of the constructed structure was 65 x 116 $Å^2$ as compared to 76 x 126 $Å^2$ for the real p21 crystal (Hebert et al., 1985b). The unit cell angles were 112^o and 96.5^o, respectively. For both structures the long axis of the central ellipsoid domains of the Na,K-ATPase molecules is approximately perpendicular to the axes connecting the units forming the $(\alpha\beta)_2$-unit. Thus, the structure has an open protein deficient region in the centre. The connectivity between Na,K-ATPase molecules is primarily located to one of the peripheral domains. This has earlier been assumed to correspond to the intracellular part of the molecule (Hebert et al., 1985). The most significant structural difference between the model derived from the p21 crystal and that calculated from the p1 crystal is located at the other end of the molecule. The peripheral domains of the two protomers come close together in the constructed pair while the $(\alpha\beta)_2$-unit shows a much more open structure. Differences in the topography of the two models cannot be accounted for by an E_1-E_2 transition since vanadate, which is proposed to stabilize the enzyme in its E_2 conformation (Glynn, 1985), was used to obtain both crystal forms. The results indicate that the Na,K-ATPase protomer may have a different structure when present in the monomeric and the dimeric form.

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LOCALIZATION OF TRYPTIC AND CHYMOTRYPTIC CLEAVAGE SITES IN α-SUBUNIT OF Na,K-ATPase

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INTRODUCTION

In its function as the catalytic protein of the Na,K-pump, the α-subunit undergoes transitions between E_1 and E_2 conformations that are coupled to cation transfer across the membrane (Jørgensen et al. 1982). Residues involved in the conformational transition have been identified by specific proteolytic cleavage. In the E_1-conformation cleavage is restricted to the N-terminal half of the α-subunit, while bonds near the middle and in the C-terminal half of the α-subunit are exposed to cleavage in the E_2-form (Jørgensen, 1975; Jørgensen et al.1982; Castro and Farley, 1979). The consequences of these splits for cation transport and catalytic properties of the protein have been examined in detail (Jørgensen et al. 1982, Jørgensen and Petersen 1985). Localization of these splits in the sequence of the α-subunit is therefore important for understanding the organization of this protein in the membrane and the mechanism of cation translocation.

To identify these cleavage points we isolated proteolytic fragments of the α-subunit from pig kidney by high resolution chromatography in $NaDodSO_4$. N-terminal aminoacid sequences of the fragments were determined in a gas phase sequenator and the specific cleavage sites were located within the sequences of the α-subunit (Ovchinnikov et al. 1986, Shull et al. 1986). Structure function relationships are discussed on the basis of the information available about the consequences of these splits for the Na,K-ATPase reaction and cation transport.

PATTERNS OF PROTEOLYTIC CLEAVAGE OF α-SUBUNIT

The substrate for the proteolytic enzymes is purified membrane bound Na,K-ATPase from outer medulla of pig or rabbit kidney (Jørgensen 1975) consisting of αß-units membrane discs with both surfaces exposed to the medium.

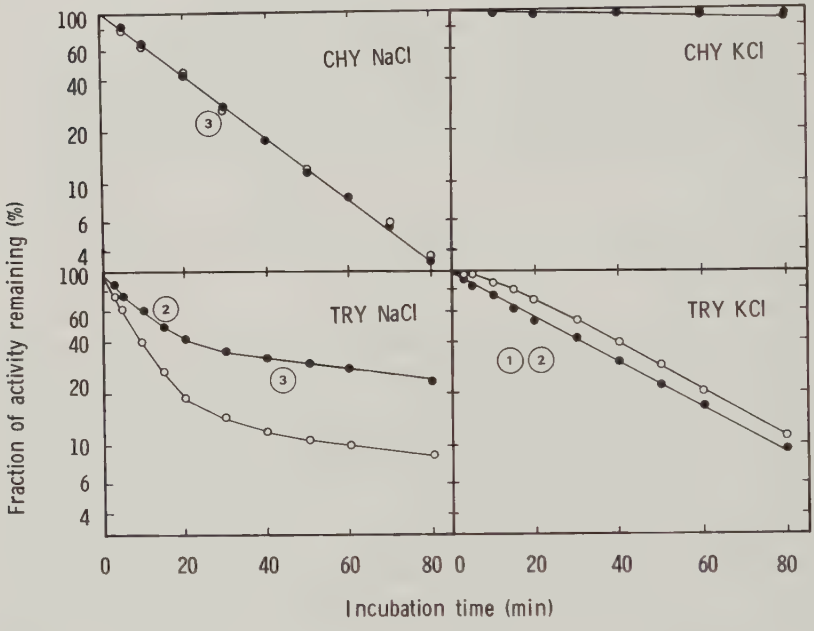

Figure 1. Time course of inactivation of Na,K-ATPase (●) or K-phosphatase (o) by chymotrypsin or trypsin in NaCl or KCl media. Procedures are described before (Jørgensen, 1975, Jørgensen et al. 1982).

In E_1-forms, bond C3 in the N-terminal half of α-subunit is cleaved by chymotrypsin A, at low ionic strength in NaCl (E_1Na) or after binding of ATP (E_1ATP) to the enzyme. As seen from Fig. 1 chymotryptic inactivation of both Na,K-ATPase and K-phosphatase is a linear function of time in semilogaritmic plots. Sites for binding of cations and nucleotides are preserved after C3 cleavage. The mechanism of inactivation appears to be that the split stabilizes both dephospho- and phospho-forms of the protein in E_1-forms.

Trypsin cleaves E_1-forms in NaCl medium (E_1Na) or after binding of ATP (E_1ATP) in two phases as seen from Fig. 1. The initial fast phase of inactivation is associated with cleavage of bond T2 near the N-terminus of the α-subunit. The second slower phase of tryptic cleavage of the α-subunit is associated with cleavage of bond T3 in the N-terminal half. The ratio of the rate constants for inactivation in the initial faster phase and the secondary slower phase is 50-60 at 20° C and only 15-20 at 37° C.

E_2-forms are stabilized by binding of K (E_2K) or by phosphorylation at low concentration of Na (E_2P). In the E_2-form inactivation of Na,K-ATPase is linear in semilogaritmic plots and correlates with cleavage of bond T1 near the middle of the α-subunit. Inactivation of K-phosphatase is delayed, because consecutive cleavage of bond T1 and bond T2 is required for inactivation of phosphatase activity.

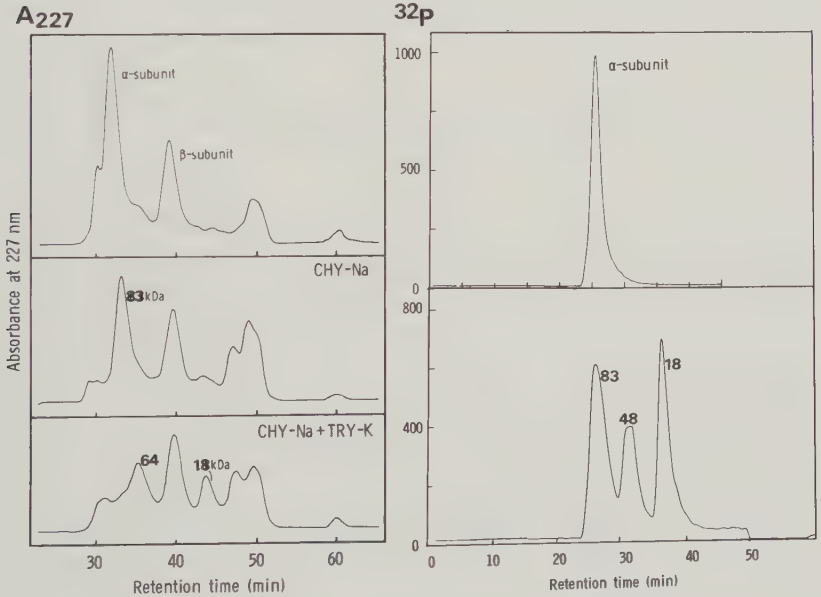

Figure 2. Separation of α-subunit, β-subunit and proteolytic fragments by HPLC chromatography on TSK 3000 SW column equilibrated with 1% $NaDodSO_4$, 100 mM sodium acetate, pH 4.5 and eluted at 0.5 ml/min. Left: absorbance at 227 nm. Right counting of ^{32}P-peptides after incorporation from ^{32}P-ATP.

All degradation rates depend strongly on ionic strength. An increase of ionic strength reduces all cleavage rates. This is an effect on the membrane protein, since the activities of trypsin towards casein or synthetic substrates are unaffected by salt.

ISOLATION OF FRAGMENTS

Proteolytic fragments can be separated with high resolution on the TSK 3000 SW column in $NaDodSO_4$ medium as shown in Fig. 2. After elution with 1% $NaDodSO_4$ at pH 4.5 the α-subunit, β-subunit, 83 kDa fragment, and 18 kDa fragment appear as separate peaks of peptides with well defined N-terminal sequences. In contrast, isolation of the fragments arising by tryptic cleavage near the middle of the α-subunit poses problems. The two fragments are almost of equal size, 64 and 48 kDa, but the C-terminal fragment of 64 kDa was eluted earlier than the β-subunit, while it was difficult to isolate the 48 kDa, N-terminal fragment.

N-TERMINAL SEQUENCING OF FRAGMENTS

Aminoacid sequences were determined on an Applied Biosystems Model 470 gas phase Protein Sequencer using standard procedures (see Jørgensen and Collins, 1986).

N-terminal sequences of fragments with M_r 82,978 and 18,722 were in both cases ASGLEGGQTPIAA, thus identifying the chymotryptic cleavage site at low ionic strength in NaCl medium (E_1-form) to the Leu-266 and Ala-267. This confirms localization of the chymotryptic split to the N-terminal half of the α-subunit that was based on experiments with phosphorylation of the fragments (Jørgensen et al 1982). The fragment with M_r 18.722 incorporates phosphate from [γ^{32}P]-ATP into the phosphorylation site, Asp-369. It also incorporates hydrophobic labels from the lipid bilayer and it participates in formation of the binding site for ouabain at the extracellular surface. This information together with the localization of the cleavage points at the cytoplasmic surface were essential features for the model of the path of the α-subunit in the membrane bilayer (Jørgensen et al. 1982).

N-terminal sequence of α-subunit is shown in Fig. 4. The N-terminal 13 residues of the purified α-subunit of Na,K-ATPase from pig kidney outer medulla correspond to those deduced from cDNA sequences of pig, sheep and rat (Ovchinnikov et al. 1986, Shull et al. 1986) when the initiating Met and first 4 residues (MGKGV) are cleaved off. After completion of the fast phase of tryptic cleavage

in NaCl medium, the N-terminus of the fragment was EVSMXXXKLSLXELXXKY thus identifying T1 cleavage to the bond between Lys-30 and Glu-31. After sequential cleavage, the 64 kDa fragment arising by tryptic cleavage of the 83 kDa fragment was eluted earlier than the β-subunit (Fig. 2). N-terminus of the 64 kDa fragment was AVAEXAXEXALL, thus identifying the T2 cleavage to the bond between Arg-438 and Ala-439.

MOLECULAR WEIGHT OF FRAGMENTS

Comparison of compositional values with molecular masses determined by gel-electrophoresis in $NaDodSO_4$ (Jørgensen 1975, 1982) shows a good correspondence for the N-terminal half of the α-subunit while the molecular masses of the C-terminal half were underestimated by gel electrophoresis, like the molecular mass of the entire α-subunit, Fig. 3. Relatively fast migration in gel electrophoresis with underestimation of the molecular mass of the α-subunit may thus be related to abnormal binding of $NaDodSO_4$ or incomplete unfolding of segments within the C-terminal half of the α-subunit.

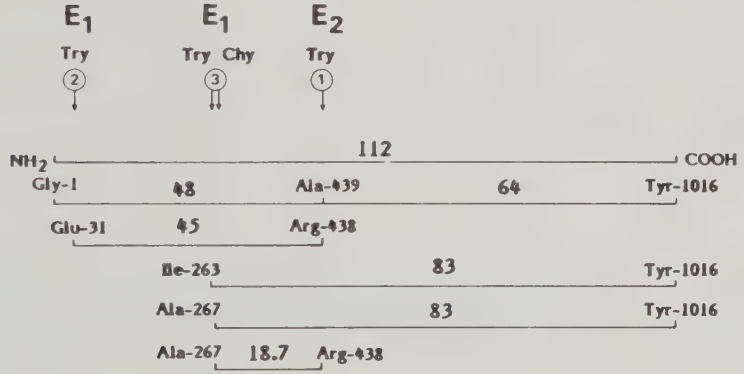

Figure 3. Molecular masses of proteolytic fragments of the α-subunit.

Control (C) or cleaved (T2) preparations of membrane-bound Na,K-ATPase incorporated identical amounts of fluorescein-isothiocyanate covalently. It was therefore possible to titrate the effect of cleavage of bond T2 on transition from E_2K to E_1Na at different values of pH. Titration of the fluorescence responses with NaCl shows that $K_{0.5}(Na)$ was decreased when pH was raised. At high pH (7-8.8), $K_{0.5}(Na)$ was only slightly higher for control than in cleaved preparations. At low pH (5.9-6.2) a larger difference in

$K_{0.5}$(Na) became apparent. In the range of pH values from 5.9 to 6.3 $K_{0.5}$(Na) of the cleaved preparation was significantly lower than for control (Jorgensen and Collins 1986). Removal of 30 residues of the N-terminal end of the α-subunit thus increases the apparent affinity of the enzyme for Na. This result suggests that removal of an ionizable group with pK near 6, e.g. a histidine (His-13) may be responsible for the increase in affinity for Na.

UNIQUE CLEAVED DERIVATIVES OF Na,K-ATPase

Selective cleavage of T2 and C3 in α-subunit of Na,K-ATPase is important for examining structure-function relationships for the protein. With the proper selection of experiments it is possible to distinguish interference with ligand binding from effects on conformational E_1-E_2 equilibria in the protein.

REGULATORY FUNCTION OF N-TERMINUS OF Na,K-ATPase

The N-terminus of the α-subunit is a strongly hydrophilic and flexible structure with a strong propensity for α-helix formation and several predicted turns, notably at residues 14-16, 35-36, 49-50 and 70-72. Selective tryptic cleavage at Lys 30 (T2) is possible because its cleavage rate is up to 60 fold higher than the rate of tryptic cleavage at Arg-262 (T3) in the second slow phase of inactivation. Removal of residues 1-30 reduces Na,K-ATPase activity and Na,K-transport by 50-60%. The loss of activity is

```
                                                    T2
                                                    ↓
Pig α          MGKGV-GRDKYEPAAVSEHGD--KK--KAK-KERDMDELKKEVSMDDHKLSLDELHRKY
Rat α          MGKGV-GRDKYEPAAVSEHGD--KKSKKAK-KERDMDELKKEVSMDDHKLSLDELHRKY
Rat α+         MGRGA-GRE-YSPAATTAENGGGKK--KQK--EKELDELKKEVAMDDHKLSLDELGRKY
Rat α-III            MGDKKDDKSSP--KKS-KAK-ERRDLDDLKKEVAMTEHKMSVEEVCRKY
Torpedo α      MGKGA-ASEKYQPAATSENA---KNSKKSKSKTTDLDELKKEVSLDDHKLNLDELHQKY
H,K-ATPase     MGKENYELYSVELGTGPGGDMAAKMS-KKKA--GGGGGKKKEKLENMKKEMEMNDHQLS
Ca-ATPase      MEAAHSKSTEECLAYFGVSETTGLTPDQVKRHLEKYGHNELPAEEGKSLWELVIEQFED
```

Figure 4. N-terminal sequence of α-subunit with localization of bond T2 (Lys 30-Glu 31). Comparison of α-isoform from pig with α, α+, and α-III from rat brain (Shull et al. 1986), Torpedo α-isoform, H,K-ATPase and Ca-ATPase (For ref. se Jørgensen, this volume).

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explained by a change in poise of equilibria between cation bound (E_1Na-E_2K) and phosphoforms (E_1P-E_2P) in direction of E_1-forms These properties of the selectively cleaved derivative suggested that charged residues in the N-terminus engage in salt bridge formation

as part of E_1-E_2 transition (Jørgensen et al. 1982) and that this is important for control of the rate of active Na,K-transport.

In agreement with this, substantial differences in sequence and number of charged residues between aminoacid sequences of α-, α+, and α-III isoforms are found in the N-terminal region (Shull et al. 1986). The different properties of the isoforms may be explained by differences in rates of E_1-E_2 transitions (Sweadner, 1985). Our data therefore suggest that the N-terminus is important for regulation of the rate of Na,K-transport.

Also the N-terminus of H,K-ATPase (Shull and Lingrell 1986) possesses a lysine-rich sequence with strong homology with the α-subunit around T2. Biphasic cleavage patterns resembling those in Na,K-ATPase have also been demonstrated in H,K-ATPase (Saccomani et al. 1981), but so far without identification of cleavage points.

EFFECT OF C3 CLEAVAGE ON E_1P-E_2P TRANSITION AND CATION EXCHANGE

C3-cleavage is a selective and particularly efficient tool for examining structure-function relationships of the second cytoplasmic domain. Binding affinities for ADP and ATP are reduced 4-5 fold, while TNP-ATP and eosin bind with the same affinity as in native Na,K-ATPase (Jørgensen and Petersen 1985). Nucleotide binding is not affected by K^+ or Rb^+ although cation sites are undamaged. Conversely the cleaved enzyme also binds ^{86}Rb with high affinity and it occludes the cations, but binding and occlusion are unaffected by nucleotide. The alternating exposure of T1 (Arg-438) in the E_2-form and C3 (Leu-266) or T3 (Arg-262) in the E_1-form reflects that motion within the segment (M_r 18,170) between these bonds including the phosphorylated residue (Asp-369) is an important element in E_1-E_2-transition.

Combination of C3 and T1 cleavages allowed comparison of the properties of four phosphopeptides, the intact α-subunit, and the 83 kDa, 47 kDa, and 18 kDa fragments, Fig. 1 (Jørgensen et al. 1982). Transfer of phosphate from ATP to the fragments remains sensitive to Na^+ but the 87 kDa and 18 kDa fragments are stabilized in the E_1P-form. This is a specific effect of C3 cleavage, because comparison with the properties of the 47 kDa fragment shows that after cleavage of T_1 alone, the 47 kDa fragment forms both E_1P and E_2P and the Na-Na and K-K exchange reactions are preserved.

Thus, C3 cleavage blocks the relatively fast Na-Na or K-K exchange (20-40 s^{-1}) and Na-K exchange (500 s^{-1}), but the slow

passive ouabain-sensitive Rb-Rb-exchange (1 s^{-1}) and occlusion of K^+ or Na^+ are hardly affected. This is basis for the conclusion that C3 cleavage interferes with coordination between structural changes of the phosphorylated segment and cation sites that may be formed by segments in the second cytoplasmic domain of α-subunit (Jørgensen et al. 1982).

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STRUCTURE AND FUNCTION OF THE β SUBUNIT OF (Na,K)ATPase

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INTRODUCTION

(Na,K)ATPase has been shown to consist of two non-covalently linked polypeptide subunits, a larger catalitic α subunit of about 110KDa and a glycosylated β subunit of about 35KDa, in a equal molar ratio. Hitherto all physiological features of the enzyme, such as location of the binding site for ATP and ouabain and the phosphorylation site in an intermediary reaction step, have been attributed to the α subunit. For the β subunit, no positive evidence indicating a definite role in ATP hydrolysis or in ion transport function has been demonstrated. However, the fact that the reduction of the disulfide bond located in the β subunit results in the total inactivation of the enzyme implies that the β subunit is essential at least to maintain the enzyme structure[Kawamura & Nagano, 1984; Kawamura et al., 1985].

We describe here further evidence for the absence of disulfide bonds in the α subunit and for the location of a disulfide bond in the primary structure of the β subunit.

MATERIALS AND METHODS

(Na,K)ATPase was purified from dogs' kidney outermedulla by the method of Jorgensen[Jorgensen,1974]. To avoid spontaneous oxidation of SH groups during purification, 0.1 to 1mM dithiothreitol(DTT) was used throughout the purification.

The number of disulfide bonds was estimated from the

number of SH groups newly produced by reduction of the enzyme whose intrinsic SH groups had been alkylated with N-ethylmaleimide(NEM). The prealkylation was carried out in 1% SDS containing 1mM EDTA, 50mM Tris/HCl(pH 8.0), 1mM NEM and 4 to 5mg/ml protein for 2h at room temperature. A rather higher pH was employed in order to achieve complete modification of the intrinsic SH groups. The excess of NEM was removed by dialysis against 0.1% SDS containing 0.1mM EDTA and 5mM Tris/HCl(pH 8.0). The alkylated protein was then reduced under the same conditions for alkylation except NEM was replaced by 20mM DTT. After standing overnight at room temperature the alkylated and reduced protein was dialyzed against deairated 0.1% SDS containing 1mM EDTA and 50mM phosphate buffer(pH 7.5). SH groups produced by the reduction were assayed radiochemically with 1mM 2,3- ^{14}C-NEM (2.4mCi/mmol).

Protein was determined by the method of Lowry with bovine serum albumin from Armour Pharmaceutical Company as a standard.

RESULTS AND DISCUSSION

Since (Na,K)ATPase contains a large number of intrinsic SH groups(for example, 30 SH groups for Torpedo californica enzyme), estimating the number of disulfide bonds as the additional increase in SH groups due to reduction was unreliable. We, therefore, prealkylated the intrinsic SH groups with cold NEM and then reduced the resulting alkylated protein with DTT. The SH groups newly produced were labeled with 2,3-^{14}C-NEM.

The labeled protein was separated by Sepharose 4B column chromatography. The typical elution pattern from the column is shown in Fig.1. The fractions corresponding to the α and the β subunit were pooled as indicated by bars in Fig.1, and analyzed for protein and radioactivity. The numbers of SH groups were calculated to be 3.5 and 4.1 mol per mol of the α and the β subunit, respectively.(Molecular weights of the subunits were assumed to be 110000 for the α and 35000 for the β subunit.) These results meant that there were 1.5 and 2.8 mol of disulfide bonds in the α and the β subunit, respectively.

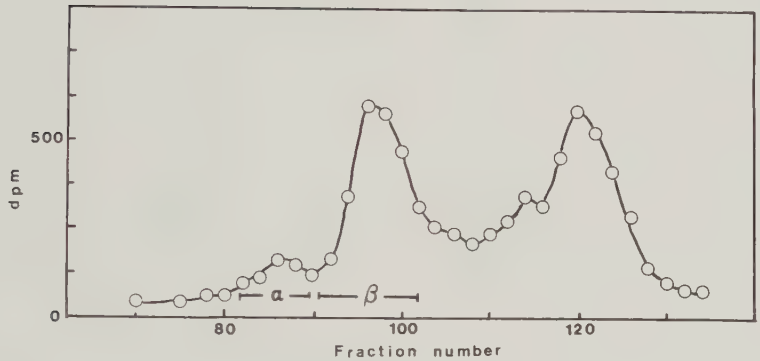

Fig.1. Sepharose 4B chromatography of (Na,K)ATPase labeled with ^{14}C-NEM. (Na,K)ATPase was prealkylayed with cold NEM and reduced with DTT. The reduced enzyme was then labeled with ^{14}C-NEM and applied on a Sepharose 4B column. An aliquot of each fraction was counted for radioactivity.

However, when the pooled fraction for the α subunit was assayed by SDS polyacrylamide gel electrophoresis, there was little radioactivity on the α subunit band but a fair amount of radioactivity near the dye front was found. In the case of the β subunit fraction, most of the radioactivity remained at the β subunit band and only a small amount of the radioactivity was released to the dye front(Fig.2). Since both the α and the β subunit were stained with Coomassie blue at the corresponding positions on the gels, the release of radioactivity was not likely due to the proteolysis of the subunits.

A large portion of the radioactivity released to the dye front was lost from the gels during the staining and destaining procedure, which suggested that the radioactivity was associated with a low molecular weight component(Fig.2). The component was, however, neither so small as to be permeable through dialysis tubing nor dissociable from the subunits during gel filtration in 0.1% SDS.

The component was not likely to be a small peptide, since when the component was amino acid analyzed, no significant amount of amino acids were detected(data are not shown). The possibility that the component might be the proteolipid subunit of (Na,K)ATPase reported by Reeves et al.[Reeves et al., 1980] could be excluded.

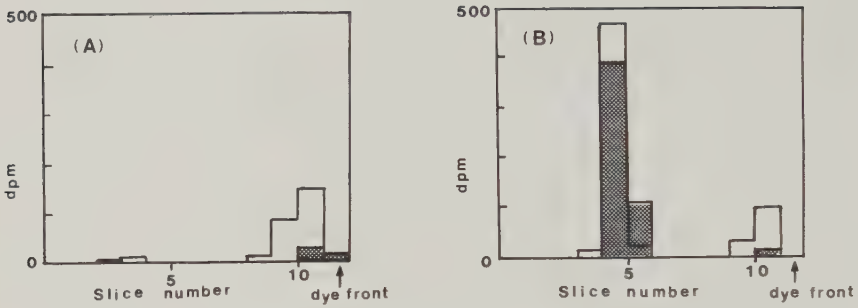

Fig.2. Release of radioactivity from the subunits of (Na,K)ATPase upon electrophoresis. The α and the β subunit fractions in Fig.1 were analyzed by SDS gel electrophoresis. The radioactivity in gel slices was measured with (histograms) or without (shadowed histograms) staining and destaining procedure. (A): α subunit fraction, (B): β subunit fraction.

The most possible candidate for such a low molecular weight component was phospholipids. The following observations supported this supposition : (i)azolectin could be modified with NEM(0.05 μmol NEM/mg azolectin) when it was treated with DTT prior to the alkylation, (ii)phospholipids were electrophoresed near to the dye front on SDS polyacrylamide gels.

Why could phospholipids be dissociated from proteins by gel electrophoresis and not by gel filtration, if the component was phospholipids? For gel electrophoresis, SDS is usually used at a concentration of 1% to solubilize proteins and the ratio of SDS to protein is very high. At such a high SDS/protein ratio, even the phospholipids firmly attached to intrinsic proteins could be dissociated substantially from the hydrophobic surface of the proteins. This might be the reason for the release of the radioactivity from the subunits of (Na,K)ATPase by SDS gel electrophoresis. Therefore, we treated the α and the β subunit fractions from the column shown in Fig-1 with 5% SDS at higher SDS/protein ratio and the resulting subunit fractions were re-chromatographed on a Sepharose 4B column. As expected, the radioactivity was dissociated from the subunits, largely from the α subunit, and eluted out from the column at low molecular weight factions where a broad peak of phospholipids was usually

observed(data are not shown). We, then, recalculated the number of disulfide bonds based on the radioactivity still remaining in the subunits whose phospholipids had been split off by treatment with a high concentration of SDS followed by column chromatography. The results were 0.3mol(n=5) and 2.3 mol(n=4) NEM per mol of the α and the β subunit, respectively. These results together with the result shown in Fig. 2(A) indicated that there was no disulfide bond in the α subunit. For the β subunit, we could not draw a conclusion on the number of disulfide bond, since the β subunit fraction was often contaminated by the fragment of the α subunit. Therefore, the data seemed to suggest the presence of one to two disulfide bond within a molecule of the β subunit.

We have reported[Kawamura & Nagano, 1984; Kawamura et al., 1985] that the reduction of disulfide bond(s) of (Na,K)ATPase results in the total inactivation of the enzyme, and that from the results of fluorescent chemical labeling experiments the reduced disulfide bond(s) is in the β subunit . The present result that the α subunit lacks a disulfide bond, strongly supports these conclusions.

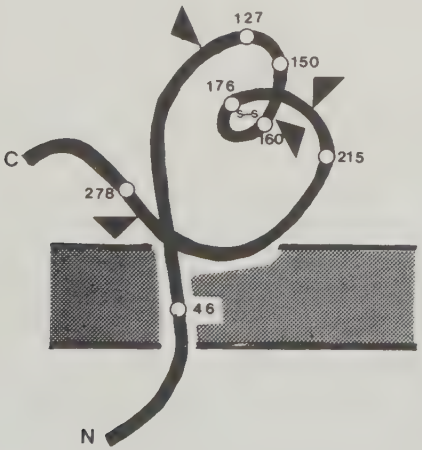

Fig.3. A model for the β subunit. The locations of disulfide bond and of cysteine residues(circle) and four possible glycosylation sites are drawn on the sequence of the Torpedo β subunit.

The β subunit has seven cystein residues in its primary structure[Noguchi et al., 1986]. To determine which cystein residues are involved in the formation of the disulfide bond(s) is a important problem. During the course of the study on the structure of the extra-membranous domain of the β subunit with Dr. Ohta, we have found a tryptic peptide containing a disulfide bond which cross-links cystein 159 and 175[Ohta et al., 1986].

Based on the hydropathic profile of the primary structure of the β subunit together with the results on the disulfide bond(s), a possible model for the structure of the β subunit is proposed and is shown in Fig. 3.

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THREE-DIMENSIONAL STRUCTURE OF Na,K-ATPase AND A MODEL FOR THE OLIGOMERIC FORM AND THE MECHANISM OF THE Na,K PUMP

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INTRODUCTION

In our laboratory we have conducted structural studies of the Na,K-ATPase by electron microscopy and image analysis of its crystalline sheets. We also have designed and performed biochemical experiments to complement the structural investigations. Initially we demonstrated that in addition to the intact molecule, which had been crystallized by Skriver et al. (1981), it was possible to crystallize preparations of a trypsin-digested form of the enzyme in which a fragment of M_r 26,000 had been removed from the N terminus of its α subunit. This corresponded to the 'Na form' described by Jorgensen (1975). Computer filtration of negatively stained images of the intact crystalline enzyme showed that, in projection, the molecule was composed of a massive 'body' with a less massive 'hook' extending from it. Comparison of these images with those of the digested enzyme indicated that the mass lost by proteolysis had been removed from the 'body' of the enzyme molecule. This allowed the identification of at least a portion of the 'body' with the α subunit. Furthermore, comparison of the shape of the molecule and the unit cell dimensions in the crystalline sheets of Na,K-ATPase with the corresponding parameters in the Ca-ATPase (e.g. Buhle et al., 1983) indicated that the 'hook' region might accommodate the ß subunit (Mohraz and Smith, 1984; Mohraz et al., 1985a). Subsequent reconstruction of the three-dimensional (3-D) structure of the enzyme has provided further support for these assignments (Mohraz et al., 1987). In an effort to

produce better-ordered crystalline sheets suitable for 3-D structural studies, we discovered that incubation of the enzyme with phospholipase A_2, with concurrent dialysis against a buffer that contained crystal inducing-ions, caused extensive crystalline arrays to form overnight (Mohraz et al., 1985b). These arrays were exclusively dimeric with an $(\alpha\beta)_2$ structure in the unit cell (Fig. 1). Examination of the crystallization process showed that pairs of ATPase molecules initially interacted to form long ribbons. These ribbons, which formed as soon as incubation with phospholipase started, subsequently associated laterally to form the extended arrays. Inspection of the ribbons revealed that the initial interaction in the pair of enzyme molecules was between the domains that we had identified as the α subunits. Finally, biochemical studies of dimeric crystalline sheets have yielded results with implications for the oligomeric form of the functional unit and suggestions as to how the transport of Na^+ and K^+ might occur (Mohraz et al., 1986).

THE THREE-DIMENSIONAL STRUCTURE

Tilt series were recorded at 60, 55, 50, 45 and 30 degrees from negatively stained specimens of phospholipase induced dimeric sheets of the enzyme, similar in quality to the sheet shown in Fig. 1.

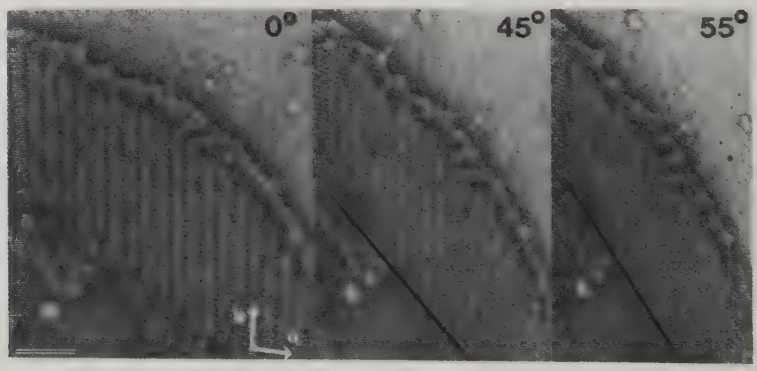

FIGURE 1. An example of a typical tilt series. The diagonal lines indicate the direction of the tilt axis with respect to the specimen. The white arrows indicate the direction of the lattice vectors. Bar, 100nm

A total of seven tilt series were computer filtered, and the resulting 42 independent projection images were used in the computation of the 3-D map. The final reconstruction, computed to an overall resolution of 2.5nm, showed high contrast structural features only in a limited volume. This was a region of approximately 6.0nm in depth, abutting one side of the membrane. We attribute the structure to the cytoplasmic side, since most of the mass of the molecule is known to be located on this surface of the membrane (e.g. Ohta et al., 1986), and little structure could be seen on the exoplasmic side by freeze-drying and metal-shadowing (Mohraz et al., 1984). The limitation of recoverable information to one side of the membrane has been a common finding in 3-D studies of membrane proteins from negatively stained specimens, for example in the bladder membrane (Brisson and Wade, 1983) and in the Ca-ATPase (Taylor et al., 1986).

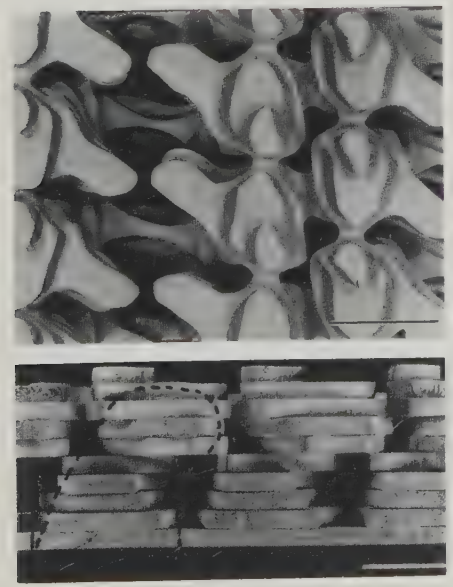

FIGURE 2. Balsa wood model of the mass of the enzyme that projects from the plasma membrane into the cytoplasm. Upper panel shows the view from within the cell normal to the membrane. Bar, 5nm. Lower panel shows a side view of the model. The dotted line demarks one molecule and shows that it is tilted towards the membrane. The black band at the bottom of the figure indicates the approximate position of the plasma membrane. Bar, 2.5nm. Key to the structural features is provided in Fig. 3.

Fig. 2 shows a balsa wood model of the reconstructed 3-D structure. The molecule has a massive 'body' that is tilted with respect to the membrane (lower panel). A 'lobe' extends from the body in parts of the structure furthest from the membrane, and an 'arm' projects from it

in the region adjacent to the membrane (see Fig. 3). The structural features correspond to those we had seen in the projection images of the molecule; however, in the latter the arm and the lobe appeared superimposed and we termed the structure the 'hook'. Within the ribbon, bridges are made between the enzyme molecule and its neighbours. The first link is the one that interconnects the two monomers to form the dimer. This bridge, which is shown in Fig. 3, is located at a distance of 3.5nm above the membrane. It connects the domains previously identified as the α subunits, based on the comparison of the projection images of the intact and the digested enzyme (Mohraz and Smith, 1984). The trypsin digestion scheme had specifically removed a segment from the amino terminal of the α subunit. Since we have never succeeded in forming dimeric arrays in the digested preparations of the enzyme, we conclude that the interaction responsible for dimer formation is between segments within the amino termini of the α subunits. The ribbons themselves are interconnected by the arm structures that are located adjacent to the membrane. The arms (hooks) were tentatively ascribed in the projection images to the ß subunits. Since ß protrudes only slightly from the cytoplasmic side of the membrane, the finding that these bridges are close to the membrane tends to support the earlier identification.

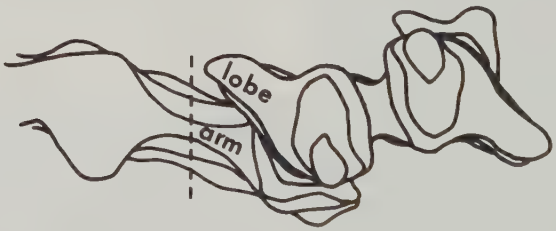

FIGURE 3. Isodensity contour map, viewed normal to the membrane. For simplicity only the middle molecule shows the whole volume of reconstruction, with partial views being shown of the adjacent molecules to emphasize the location of the bridges. The structures labeled the 'lobe' and the 'arm' project from the tilted column of mass that we have termed the 'body'. The structure to the right of the dotted line is the dimer and the linkage between the two molecules is attributed to an α-α interaction. The inter-ribbon bridge is formed by the arms and is tentatively identified as a ß-ß interaction.

CONFORMATION OF Na,K-ATPase IN THE DIMERIC SHEETS

Jorgensen (1975) showed that limited digestion of membrane-bound Na,K-ATPase by trypsin in the presence of Na^+ and in the presence of K^+ produced different cleavage patterns, indicating that the enzyme assumed different conformations depending on whether it had bound Na^+ (E_1) or bound K^+ (E_2). The gel in Fig. 4 shows the pure enzyme in lane A, and its trypsin-digested patterns in lanes B (E_1, Na form) and C (E_2, K form). In the 'Na form', the α subunit is cleaved to a fragment of M_r 77 kd without apparent digestion of the ß subunit. In the 'K form', the α subunit is digested to produce the doublets at M_r 58 kd and M_r 41 kd.

We exploited the specificity of trypsin digestion to investigate the conformational state of the ATPase molecule in the dimeric arrays. Dimeric sheets of the enzyme, induced by phospholipase A_2 and various inducing ions were digested with trypsin. Na^+ and K^+ were excluded from the proteolysis buffer so that the conformation of the enzyme in the crystalline state would not be disturbed. The gel in Fig. 4, lane D shows the fragmentation pattern of the dimeric sheets of Fig. 4D (micrographs), which were induced in the presence of PO_4^{3-} and Mg^{2+}. Dimeric sheets induced by phospholipase A_2 and other ions also showed a similar proteolysis pattern. The pattern corresponded to a combination of the 'Na' and the 'K' forms: it had both the 77 kd piece and the doublets at 58 kd and 41 kd, implying that, in the dimeric sheets, both conformations of the enzyme were present. In order to be sure that this unexpected fragmentation pattern was not caused by phospholipase A_2, we treated the purified enzyme with phospholipase A_2 under ionic conditions that locked the enzyme molecules either in the E_1 or in the E_2 conformation (see the figure caption for experimental conditions). Fig. 4E and 4F (micrographs) show the E_1 and the E_2 preparations, respectively. We never saw evidence of dimeric sheets, or even ribbons, in either of the two specimens. In the E_1 conformation (4E), the molecules were either close-packed or formed rope-like structures that resembled beads on a string. On rare occasions, monomeric type arrays similar to the one shown at the lower half of the figure were observed. In the E_2 conformation (4F), the molecules were either close-

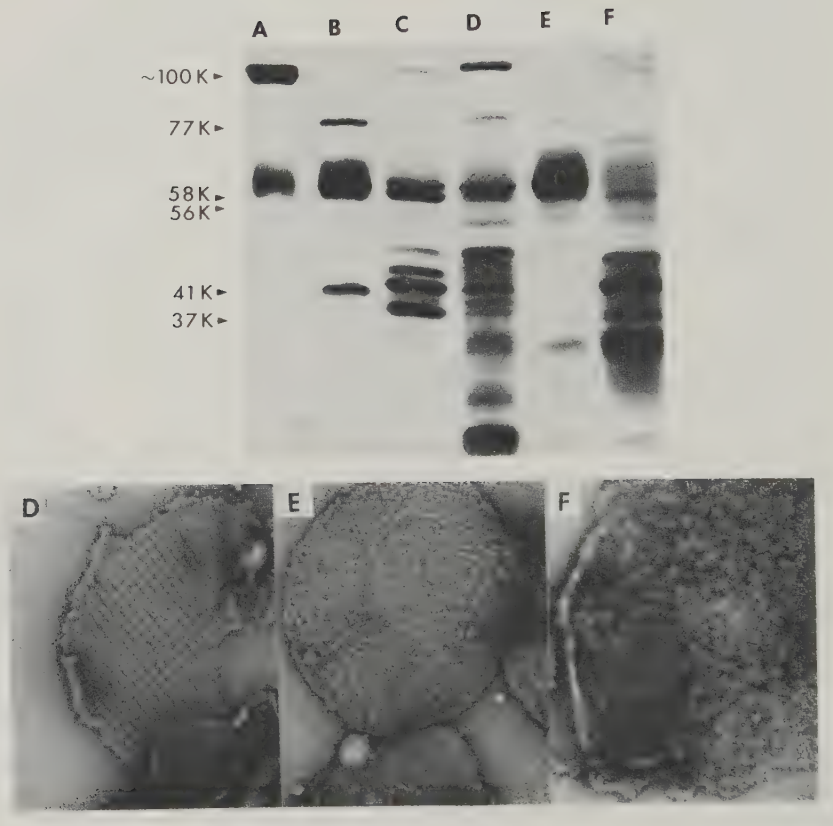

FIGURE 4. Upper panel shows the trypsin digestion of Na,K-ATPase sheets. Lane A, purified enzyme; B, 'Na form' digestion of the purified enzyme; C, 'K form' digestion of the purified enzyme; D, dimeric sheets of the enzyme induced by phospholipase A_2, Mg^{2+}, PO_4^{3-}, digested with trypsin. The fragmentation pattern corresponds to a combination of the 'Na' and the 'K' forms; E, enzyme sheets treated with phospholipase A_2, and Na^+ (E_1 conformation), digested with trypsin. The pattern corresponds to the 'Na form' digestion; F, enzyme treated with phospholipase A_2, Mg^{2+}, PO_4^{3-} and ouabain (E_2 conformation), digested with trypsin. The proteolysis pattern is similar to the 'K form'. Lower panel shows micrograghs of the specimens whose digestion patterns are shown in lanes D, E, and F of the gel, respectively.

packed or formed small monomeric crystalline arrays. Trypsin digestion of the sheets produced the expected 'Na form' and the 'K form' patterns, respectively. These are presented in the gel of Fig. 4, lanes E and F. The possibility that in the dimer the molecule could assume a conformation in which both trypsin sensitive sites are exposed simultaneously is excluded, since if this were the case, the digestion pattern would not show the bands at 77 kd and 58kd, but instead fragments of lower molecular mass.

A MODEL FOR THE OLIGOMERIC STRUCTURE AND MECHANISM OF THE Na,K PUMP

These results have implications for the oligomeric structure as well as the mechanism of the Na,K pump. They suggest that the dimer is formed by interaction of two ATPase molecules, one in the E_1 (Na binding) and the other in the E_2 (K binding) conformation. Although this does not provide direct evidence that the dimer is the functional unit, formation of extensive and very stable crystalline arrays, in which a dimer of conformationally distinct molecules constitutes the building block, does imply functional significance.

If the dimer is the functional unit, then the pump must operate in a manner that is shown schematically below:

In this model α subunits interact to form the dimer. During the pumping cycle, the two halves of the unit operate 180° out of phase such that while one molecule is in the E_1 conformation and pumps Na^+ out of the cell, the other is in the E_2 state and transports K^+ into the cell. By the end of the cycle the two would have exchanged conformations. This model is closely related to the "flip-flop" model hypothesized by Stein et al. (1973), Repke and Schon (1973), and Repke and Dittrich (1979).

Support for the dimer as the active unit comes from numerous studies. However, a number of other studies,

mainly of the detergent-solubilized enzyme, have pointed to the protomer as the functional unit (for a review see Glynn, 1985). To date, the nature of the oligomeric structure of the pump remains a subject of controversy. The existence of a dimer of conformationally distinct molecules represents the first structural result in support of a mechanism of transport where the two halves of the dimeric pump function out of phase.

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STRUCTURE OF Na,K-ATPase: THE LOCATIONS OF ATP BINDING REGIONS, TRYPSIN ACCESSIBLE REGIONS AND MEMBRANE-EMBEDDED REGIONS

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INTRODUCTION

Substantial progress on Na,K-ATPase has been made in recent years in establishing the sequences of the enzyme subunits (Shull et al., 1985; Noguchi et al., 1986). The use of group-specific chemical modifying agents has enabled us to identify the specific amino acid residues involved in the binding sites for the ligands of the sodium pump. Two reactive amino acid residues have been known in the active site of Na,K-ATPase: an aspartic acid phosphorylated with ATP (Walderhaug et al., 1985) and a lysine which is labeled with FITC (Farley et al., 1984; Ohta et al., 1985).

In this report, a third functional amino acid residue in the active site is determined. Extra-membranous and membrane-embedded regions are also reported. Allocation of these regions on the α- and β-subunits allows us to construct a plausible model of Na,K-ATPase and opens some new perspectives for molecular approach.

RESULT AND DISCUSSION

FSBA-Binding Site

A third functional site was confirmed in dog kidney enzyme using 5'-(p-fluorosulfonyl)benzoyladenosine (FSBA)

(Ohta et al., 1986a). This ATP analogue shares a common inhibitory feature with FITC in that it efficiently abolishes the Na,K-ATPase activity while it affects the K-p-nitrophenylphosphatase activity less effectively (Cooper and Winter, 1980). The labeling positions of FSBA were, however, different from those of FITC.

The dog kidney enzyme (27 mg) was labeled with three doses of 0.5 mM ^{14}C FSBA at 30 min invervals. FSBA labeled α-subunit (7.5 mg protein) was purified from SDS-solubilized, labeled-enzyme with a Bio-gel A column (1.5 m), SDS was removed by dialysis for 2 days and the α-subunit digested with TPCK-trypsin (10:1 ratio to protein) at room temperature for 16 hr. Purification of the FSBA-labeled peptides was carried out by reverse-phase HPLC as shown in Fig. 1.

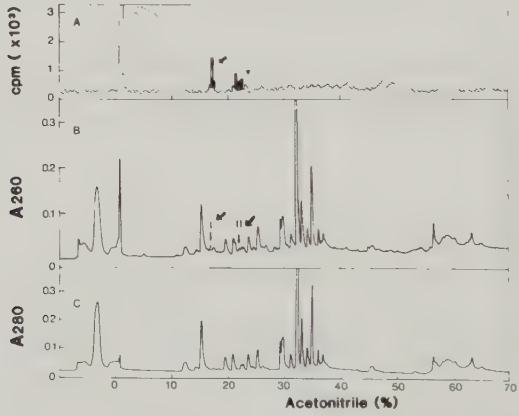

Fig. 1. Reverse-phase HPLC of tryptic peptides derived from ^{14} FSBA-labeled α-subunit of dog kidney Na,K-ATPase. The tryptic digest of the α-subunit was applied on a Bio-Rad C-4 column equilibrated in 0.1% TFA and eluted with linear gradients of acetonitrile. Radioactivity (A) and absorbance at 260 nm (B) and 280 nm (C) were monitored.

Two radioactive peak fractions (I and II) were further purified by HPLC on the same column with an extra shallow gradient of acetonitrile (0.05% per min) and sequenced on a gas phase sequencer. The sequences, Ala-X_1-Val-Val-His-Gly-Ser-Asp-Leu-Lys (peptide-I) and Gln-Gly-Ala-Ile-Val-Ala-Val-Thr-Gly-Asp-Gly-Val-Asn-Asp-Ser-Pro-Ala-Leu-X_2-Lys (peptide-II) were obtained. If the sequences of the two FSBA modified peptides are conserved in the dog and the sheep kidney and

electric organ enzymes, then the predicted amino acids in the missing cycles (X_1 and X_2) should be Cys in the peptide-I and Lys in peptide-II. Supposition of Lys as the reactive residue in peptide-II is probably valid, since Lys is known to react with FSBA to form a stable derivative whose PTH-derivative will behave differently from PTH-Lys in the analysis of the products from the Edman degradation (Colman, 1983). If the possibility of Cys is excluded, then other potentially reactive residues, such as His, Ser and Asp in the sequence, have to be considered as candidates for the labeled residue.

The importance of the two FSBA-labeled regions, especially peptide-II, is confirmed by their homology to other cation-transporting ATPases (Fig. 2). From a comparison of the entire sequence of the Na,K-ATPase α-subunit we showed the presence of three other very

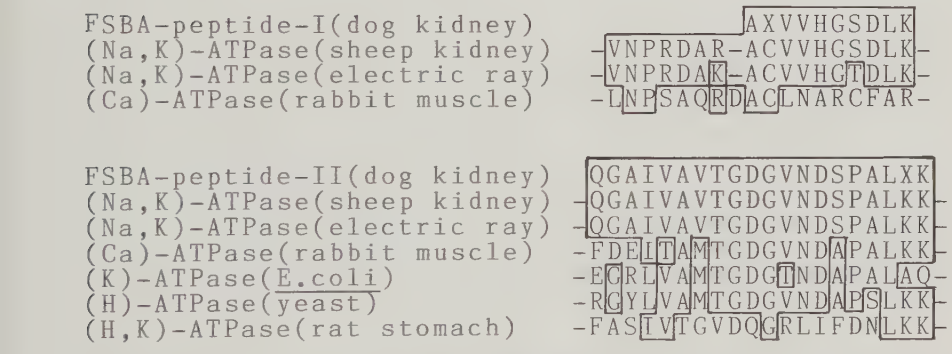

Fig. 2. Homology of FSBA-labeled peptides of dog kidney Na,K-ATPase to other ATPases. The amino acid sequences of the FSBA-labeled peptides-I and II are compared with homologous sequences of sheep kidney (Shull et al., 1985), rabbit skeletal sarcoplasmic reticulum Ca-ATPase (Maclennan et al., 1985), E. coli K-ATPase (Hesse et al., 1984), yeast plasma membrane H-ATPase (Serrano et al., 1986), and rat stomach H,K-ATPase (Shull and Lingrel, 1987). Sets of identical residues are enclosed with solid lines. X_1 and X_2 indicate the missing amino acids.

homologous regions in addition to the FITC and the phosphorylation sites. We have found that the region of peptide-

II represents one of these homologous regions and the location of peptide-I is around the middle of the two homologous regions. Information on other ATPase sequences, which is now rapidly accumulating, including sarcoplasmic reticulum Ca-ATPase. E. coli K-ATPase, yeast H-ATPase, and rat stomach H,K-ATPase reveals that considerable homology prevails among many ion transport systems beyond ion specificity and species differences. The high degree of homology of several sites may mean that they are involved in the transduction of bond energy of ATP to the energy of active transport common to these enzymes.

Extra-Membraneous Regions

Both accessibility to trypsin and facile release into the water phase indicate that corresponding peptides are derived from the exposed surface of the intact enzyme. We have sequenced some of the water soluble tryptic peptides released from the membrane-bound Na,K-ATPase of dog kidney and T. californica electric organ (Ohta et al., 1986b).

The native membrane-bound enzyme (each 2 mg) of dog and Torpedo were digested with TPCK-trypsin (50:1 ratio).The supernatant containing the soluble peptides was analyzed by reverse-phase HPLC. The peak fractions were further purified by a second run of HPLC on the same column with a less steep gradient of acetonitrile. Many peptides were released into the aqueous phase and purified to homogeneity and as many of these peptides as possible were sequenced. Fifteen tryptic peptides, 4 in the Torpedo α-subunit, 5 in the dog α-subunit and 6 in the dog β-subunit were allocated on the whole

Table 1. Sequences of the peptides released into the supernatant by trypsin digestion of the dog and electric ray membrane-bound (Na,K)-ATPase

	peptide No.	subunit	sequence
electric ray	a	α	671-DLSHENLDDILHYHTEIVF---
	b	α	386-MTVAHMWFDNQIHEADT---
	c	α	887-EKWDELWTQDLEDSYGQQWT---
	d	α	502-YLLVMKGAPER
dog	1	β	86-TLISFKPNDPK
	2	α	496-HLLVMK
	3	β	76-GLEIVPQIQK
	4	α	626-IISEGNETVEDIAAR
	5	β	112-YKDSAQKDDMIF---
	6	α	887-WINDVLDSYGQQWQYEQR
	7(1)	β	151-FKLEWLGXXSGIND---
	7(2)	β	171-EGKPXVLIK
	8	α	379-MTVAHMWFDN---
	9	β	224-FGNVEYFGLGWYPGF---
	10	α	520-EQPLDEELKDAFQ---

The number at the head of each sequence indicates the position of the amino acid of the whole sequences of the α- and β-subunit of sheep and human enzyme. X: unidentified amino acid.

sequence of human α- and β-subunit (Kawakami et al., 1986)

The sequence GXGXXG, which is characteristic of the dinucleotide binding region of various dinucleotide binding proteins, has been found in the sequence of the Torpedo β-subunit (Noguchi et al., 1986). Peptide-9 contained a similar sequence with the exception that the last glycine was repleaced by proline.

Peptide-7 was revealed to be composed of two different peptides. There are three unidentified amino acids in the sequences of peptide-7: the 8th and 9th residues in peptide-7(1) and the 5th residue in peptide-7(2). By comparing the sequence of peptide-7 with the sequence of the human β-subunit, it is likely that the 8th amino acid of peptide-7(1) is asparagine and the other two unknowns are cystein. The asparagine residue in the sequence NXS (where X can be any amino acid), well-known as the possible glycosylation site, is not recovered as PTH amino acid. The two peptides of peptide-7 behaved like a single peptide throughout the whole purification procedure. This can be explained if the two putative cysteins in peptide-7 were cross-linked by a disulfide bridge. Our result strongly suggested that peptide-7 was composed of two kinds of peptides cross-linked to each other by a disulfide bridge.

Membrane-Embedded Regions.

To establish the regions of the enzyme embedded in the membrane, several hydrophobic peptides were separated by

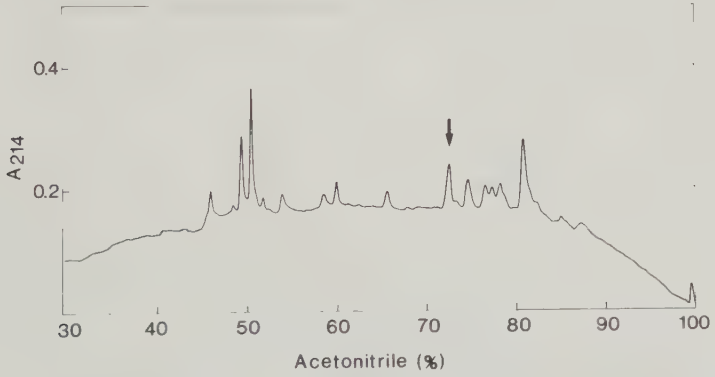

Figure 3. Reverse-phase HPLC of the membrane-embedded peptides derived from dog kidney Na,K-ATPase. The sample containing hydrophobic peptides solubilized with SDS were subjected to a Beckman C-3 column (4.6 x 75 mm) equilibrated with 0.1% TFA and eluted with linear gradients of acetonitrile.

reverse-phase HPLC.

The membrane-bound Na,K-ATPase (6.5 mg) was digested by TPCK-trypsin (25:1 ratio) at 37°C overnight and brought to pH 11.5 with 1N NaOH at 0°C and kept for 20 min. After neutralization with 1N HCl, the water soluble peptides released by trypsin were removed by centrifugation. The precipitate was solubilized with 1% SDS and analyzed through HPLC using an Ultrapore C-3 column. A few peptides were separated as shown in Fig. 3. The peak indicated by the arrow was sequenced without further purification.

Several other peaks in Fig. 3 had been lost in the process of purification possibly because of adhesion to the tube wall. The N-terminal sequence of the peptide was Ile-Ala-Thr---. The same sequence was found in the Torpedo α-subunit sequence from 270 to 272.

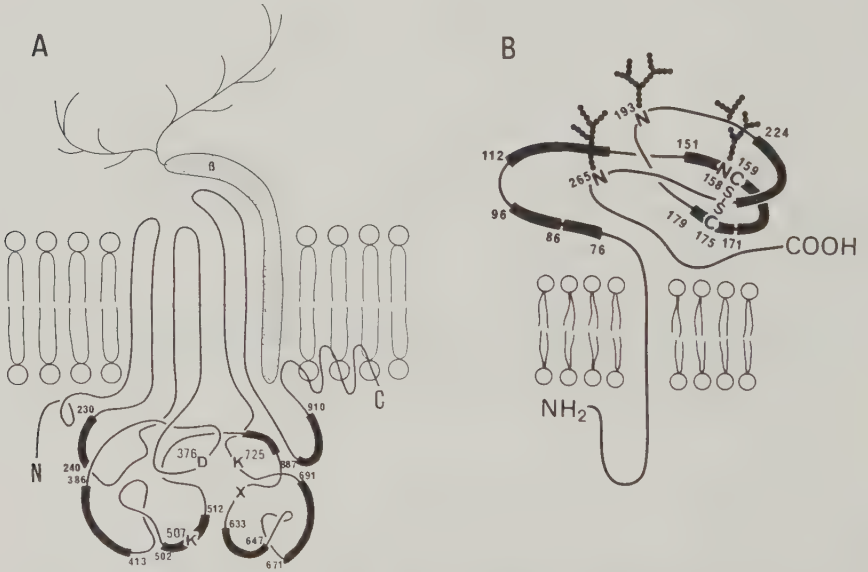

Fig. 4. Possible model for Na,K-ATPase. (A) The locations of the modified amino acid residues which are involved in the composition of active site and trypsin-released peptides were allocated to the whole sequence of the α-subunit D^{376}: phosphorylation site; K^{507}: FITC binding site; K^{725}: FSBA binding site; X: possible FSBA binding site. The out-of-membrane regions are indicated by thick lines. (B) The locations of water-soluble tryptic peptides, three possible glycosylation sites and the disulfide bridge are shown on the amino acid sequence of the human β-subunit.

Structural Model of Na,K-ATPase

Based on the results reported here together with a hydropathic profile of the primary structure of the α- and α-subunits a possible structural model of the Na,K-ATPase is proposed (Fig. 4A and 4B). Our model for α-subunit was in agreement with that (Jørgensen, 1982) which has been proposed from the results of trypsin cleavage, in the presence of KCl and NaCl, and hydrophobic labeling of the α-subunit. Our results on the active sites have contributed to the conformation model of the Na,K-ATPase α-subunit.

The model for the β-subunit based on papain digestion (Chin, 1985) differs from ours in several respects. The crucial difference is that he has postulated the existence of a second transmembrane segment in order to associate the carboxy-terminal piece with the lipid bilayer. However, if the site of papain hydrolysis is located between the cystein residues at 159 and 175, the carboxy-terminal piece can be associated with the membrane via the disulfide bridge formed between the cysteins.

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ANTIBODIES TO SYNTHETIC PEPTIDES AS PROBES OF THE STRUCTURE OF THE α SUBUNIT OF THE Na,K-ATPase

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INTRODUCTION

Since the application of molecular biological techniques has facilitated the determination of the sequences of large membrane proteins, sequences of the Na,K-ATPase from several species have been published (see Shull *et al*, this volume, for references and discussion). Analysis of the hydrophobic regions of these protein sequences (Eisenberg, 1984) predicts that the polypeptide chain might traverse the membrane eight times (but see Arzamazova *et al*, this volume). In addition, previous information about the sequence of parts of the protein-- *i.e.* the fluorescein isothiocyanate (FITC) binding site (Farley *et al*, 1984), the site of the catalytic intermediate phosphorylation (Bastide *et al*, 1973), and the sites of partial proteolytic cleavage (Jorgensen and Collins, this volume)-- has been incorporated into models of the structure of the Na,K-ATPase. Knowing the sequence of the protein makes it possible to use antibodies to synthetic peptides as tools to refine these models. To this end, we have immunized rabbits with the following peptides:

(Ac)DLDELKKEVSLDD[C](NH_2) (*Torpedo* 32-43) This sequence includes one of three clusters of charges, found between *Torpedo* α residues #34 and #55, which we speculate might be involved in ion binding. Trypsin site 2 is also within this region (Jorgensen and Collins ,this volume).

(Ac)NALTPPPTTPEWIKFC(NH_2) (*Torpedo* 78-93) This is predicted to immediately precede the first membrane-spanning

domain (See Shull *et al*, this volume, for discussion of structural predictions).

LGYTWLEA[C] (*Torpedo* 313-320) This region is predicted to be extracellular, between membrane-spanning domains 3 and 4, and possibly to form part of the ouabain binding site.

TSTICSDKTGTLT (*Torpedo* 370-382)
The aspartate in the middle of this peptide is the site of the catalytic phosphorylation (Bastide *et al*, 1973).

VMKGAPERILDRC (*Torpedo* 505-517)
FITC reacts covalently with lysine 507, totally inhibiting ATPase activity. ATP blocks this reaction , and lys_{507} is therefore thought to be in or near the ATP binding site (Farley *et al*, 1984).

Bracketed cysteine residues were added to peptides 1 and 3 for coupling purposes. Peptides are identified by their first four residues. Antibodies to these peptides have been tested for reactivity with both native and denatured *Electrophorus* (eel) membranes; some antibodies have also been tested for their ability to inhibit Na,K-ATPase activity.

METHODS

Peptides were synthesized by solid-phase methods, and were coupled via the sulfhydryl group to keyhole limpet hemocyanin using m-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) (Kitagawa and Aikawa, 1976). Rabbits were immunized at multiple sites along the back with the conjugate in Freund's complete adjuvant, and boosted monthly with the conjugate in incomplete Freund's adjuvant.

Antibodies were purified by ammonium sulfate precipitation (0-50% saturation), or by affinity chromatography. The affinity medium was prepared by coupling the appropriate synthetic peptide to AffiGel-102 (BioRad) using MBS, or by coupling solubilized eel membranes (see below) to AffiGel-102 with ethyl diaminopropyl carbodiimide. Whole serum was adsorbed to the column, the column was washed, and antibodies were eluted with 0.2 M HCl-glycine, pH 2.8 or pH 2.2. Purified antibodies were neutralized, dialyzed against tetramethylammonium (TMA) HEPES buffer, and concentrated before use. The columns were precycled with elution buffer before use.

The relative affinities of antibody for the Na,K-ATPase in native or denatured form were determined by titration of purified antibodies with eel electric organ membranes in native condition or following one minute of boiling. 10µl aliquots of purified antibody preparation were placed in 5 x 20 mm tubes, and 10µl of HEPES buffer containing various amounts of boiled or native membranes was added. The tubes were capped, mixed and incubated for 30 minutes at 37 °C. Following incubation, 180 µl of buffer was added and the tubes were centrifuged (Beckman Airfuge) for 10 minutes to sediment the bound antibody with the membranes. Antibody remaining in the supernatant was assayed by ELISA.

Eel electric organ membranes were prepared (Goldman and Albers, 1973), and then solubilized by suspending them at 10-20 mg protein/ml in 0.4 mM EDTA, 2 mM DTT, 50 mM Na-HEPES, pH 7, 20 mg/ml dodecyl maltoside, in the preparative rotor of a Beckman Airfuge for 15 min on ice, followed by 10 min centrifugation. For preparation of subunits, the soluble material was subjected to gel filtration HPLC on DuPont Zorbax GF-250 and GF 450 columns in tandem in 0.2 M Na phosphate, pH 7, 0.1 mM DTT, 0.03% dodecyl maltoside. Na,K-ATPase (the major peak) was collected, concentrated not more than two-fold by ultrafiltration, brought to 1% SDS, and rechromatographed on the same columns in 0.2 M Na phosphate, pH 7, 0.1 mM DTT, 0.1% SDS; α and β subunit peaks were collected manually. A sample of the α subunit peak was subjected to automated gas-phase sequencing.

Na,K-ATPase activity was coupled to NADH oxidation and measured spectrophotometrically (Bucher and Pfleiderer, 1955) in the presence of 60 mM Na, 20 mM K, 3 mM Mg, 200 µM ATP in 50 mM TMA HEPES, pH 7.4. Total eel membrane ATPase under these conditions is 90-95% ouabain-inhibitable.

RESULTS

Each of the five peptides stimulated the production of rabbit antiserum which reacted with the peptide, as assessed by ELISA. However, antiserum to the LGYT peptide failed to react with any preparation of eel sodium ATPase in either ELISA or Western blot assays. Antisera to the other four peptides reacted with eel membranes or purified α subunits on ELISA, and reacted specifically with the α subunit on Western blots of eel membranes run on discontinuous 6% SDS

polyacrylamide gels. Under some conditions, the α subunit band separates into two bands on these gels; all four antisera reacted with both bands. When subunits were purified by gel filtration HPLC, the peak shapes did not suggest heterogeneity, and when the intact α subunit peak was subjected to N-terminal sequencing, only one sequence was obtained. The sequence begins with serine (Perrone *et al*, 1975); the data were interpretable out to 29 cycles:
SSDEKKKDLDELKKEVALDDHKLSLTDLA
Eel #1 aligns with c-DNA-deduced #25 of *Torpedo;* strong homology begins at #31 *Torpedo*/ #8 eel.

To test whether our anti-peptide antibodies would bind to the native enzyme, the purified antibodies were absorbed out by suspensions of native (but not vesiculated) or boiled eel membranes, and the antibody remaining was measured by ELISA (Figure 1). Antibodies against TSTI and VMKG were absorbed as well by native membranes as by boiled membranes, while anti-NALT antibodies were absorbed poorly by native membranes as compared to boiled membranes. Antibodies against DLDE discriminated native and boiled membranes somewhat less well than did anti-NALT. Treatment with SDS in quantities not sufficient to solubilize the ATPase increased the ability of eel membranes to absorb anti-NALT antibodies as well as boiling did (not shown).

Affinity-purified antibodies were tested for inhibition of eel Na,K-ATPase activity. Anti-TSTI antibodies were purified on a column of DDM-solubilized eel membranes; no inhibition of ATPase by this material could be demonstrated. Anti-VMKG antibodies were purified on a column of VMKG peptide; the maximum inhibition of eel membrane Na,K-ATPase by this preparation was about 50% (Figure 2).

DISCUSSION

Antibodies to synthetic peptides are useful probes of the structure of the Na,K-ATPase. Four out of the five peptides tried so far have stimulated rabbits to produce antibodies that react with the purified eel α subunit. The epitopes represented by the various peptides vary in their accessibility in the native enzyme. Surprisingly, antibodies raised to the catalytic phosphorylation site peptide and the FITC site peptide bind almost as well to native membranes as to boiled membranes. The fact that anti-VMKG antibodies bind

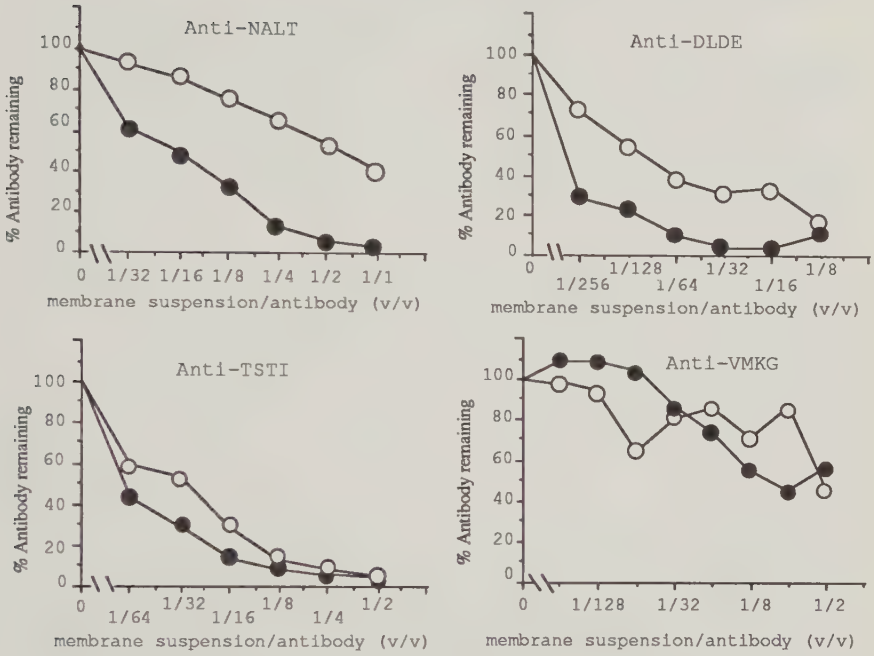

Figure 1. Absorption of Antibodies by Eel Electric Organ Membranes. Antibodies were affinity purified on columns prepared from solubilized eel membranes (anti-NALT, anti-DLDE, anti-TSTI; reconcentrated to 1/2 of the original serum volume), or purified by ammonium sulfate precipitation (anti-VMKG; concentrated to 1/2 original serum volume). Fixed volumes of purified antibody were treated with varying amounts of a suspension (8.5 mg protein/ml) of either Native (○) or Boiled (●) membranes (see Methods).

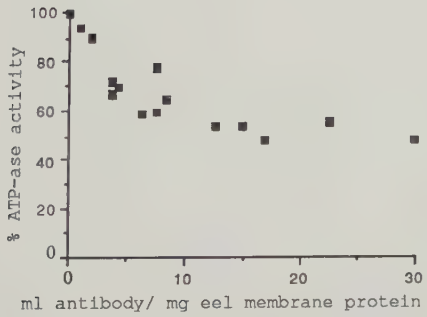

Figure 2. Effect of Anti-VMKG Antibodies on ATPase Activity. Antibody was purified on a column of VMKG synthetic peptide and concentrated to 1/3 original serum volume. Eel membranes were incubated 30 min at 37 °C with varying amounts of this preparation, then assayed for ATPase activity.

to the native enzyme and partially inhibit its activity suggests that part or all of this region of the polypeptide lies near but not directly in the ATP binding site. Why anti-TSTI antibodies do not inhibit ATPase is unknown, but this serum is of low titer, and it may contain too little antibody to have a measureable effect without further concentration.

We are developing methods to determine on which side of the membrane these and other defined epitopes lie. Preliminary results show that anti-NALT and anti-TSTI antibodies do react with tissue fixed and stained for electron microscopy.

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STRUCTURAL STUDIES OF Na,K-ATPASE SUBUNITS

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INTRODUCTION

Na,K-ATPase consists of two subunits, alpha and beta, (of molecular weights ∿120,000 and 55,000, respectively) which are found in a one-to-one stoichiometric ratio in the intact enzyme. To understand the functional roles of each subunit, it will be necessary to reconstitute chimeric molecules of Na,K-ATPase, either in vitro or biosynthetically. In that way, alternative sequences in different subunits can be correlated with different functional properties. Reconstitution of separated subunits has been accomplished for one membrane protein, S. aureus alpha-toxin, which contains a number of identical subunits (Tobkes et al, 1985); it may, however, be a more difficult task for Na,K-ATPase, which has non-identical subunits with very different solubility properties. In vitro reconstitution will require isolation of the alpha and beta subunits, then recombination of them, and finally, reassociation of the alpha/beta complexes with lipid bilayers. Observations of the conformational changes in the polypeptides at each step in the process should give insight into the folding of the polypeptides, and their refolding upon interaction with each other to form active holoenzyme.

The first step in this procedure is the separation of the two types of subunits. The alpha and beta subunits are intimately associated in the Na,K-ATPase holoenzyme, even in the absence of a lipid bilayer (i.e., when solubilized in a variety of non-ionic detergents). They can, however,

be isolated by treatment of holoenzyme with sodium dodecyl sulfate (SDS) above its critical micelle concentration (cmc), followed by SDS-polyacrylamide gel electrophoresis (PAGE) and electroelution. This paper discusses the isolation and characterization of the secondary structures of the individual alpha and beta subunits from Na,K-ATPases derived from several different animal sources.

MATERIALS AND METHODS

Purification of Protein:

Microsomes from guinea pig and dog kidney outer medulla were prepared by a modified Jorgensen (1974) procedure. Na,K-ATPase was purified according to the glycerol step gradient method (R. A. Farley, personal communication). Brine shrimp enzyme was prepared as previously described (Salon, 1986).

Isolation of Subunits:

Holoenzyme was solubilized in 2% SDS, and electrophoresed on Laemmli-type (Laemmli, 1970) gels containing 7.5% acrylamide. Alpha and beta subunit bands were cut out, and subjected to electroelution and electrodialysis, as described by Hunkapiller et al (1983). Electroelution was into a buffer containing 0.1% SDS and the final electrodialysis was into a 0.01M ammonium bicarbonate buffer, pH 7.7, containing either 0.01 or 0.02% SDS.

Spectroscopic Analyses:

Circular dichroism (CD) spectra were obtained using an AVIV 60DS spectropolarimeter fitted with a variable position photodetector, using a geometric configuration which resulted in a light accceptance angle of > 90 degrees. Spectral points over the wavelength range from 300-185 nm were taken at 0.2 nm intervals. At least 4 spectra were accumulated and averaged for each sample. In general, duplicate runs of 2 independent preparations of each sample, with concentration in the range of $\sim$ 0.5-3 mg/ml were examined in a 0.01 cm pathlength cell. The sample temperature was 25°C. Samples for baseline subtractions contained identical buffer (0.01 M ammonium bicarbonate, pH 7.7) and detergent (0.01 or 0.02% SDS) concentrations, but no protein.

The secondary structures of the isolated subunits and holoenzyme were estimated from the CD data using a linear least squares algorithm (Chang et al, 1978) without constraining the solution to sum to unity, but normalizing the total to 1.0 (Mao et al, 1982); this procedure eliminates the requirement for an absolute concentration determination (Wallace & Teeters, 1987).

RESULTS AND CONCLUSIONS

Isolation of Subunits:

The alpha and beta subunits of guinea pig Na,K-ATPase were separated by treatment with concentrations of SDS above the detergent cmc, followed by SDS-PAGE, electroelution and then electrodialysis into a buffer containing sub-cmc levels of SDS. When rerun on SDS-PAGE (Figure 1), the subunits appear as essentially single bands, even on overloaded gels. Their purity was confirmed by amino acid sequencing, which showed the bands were 80-90% homogeneous. The sum of the amino acid analyses of the alpha and beta subunits indicated a composition similar to that found for the purified guinea pig holoenzyme (Gresalfi & Wallace, 1984). The subunits were originally exposed to 2% SDS to effect the separation, but the SDS concentration could subsequently be lowered to 0.01% during electrodialysis, while still maintaining the subunits in solution.

Similar results were obtained for dog and brine shrimp enzymes, except the brine shrimp had two alpha bands, $alpha_1$ and $alpha_2$, which were individually isolated.

Figure 1: SDS-PAGE of separated Na,K-ATPase alpha (α) and beta (β) subunits isolated from dog (D) and guinea pig (G) kidneys.

Secondary Structures of Isolated Subunits:

The secondary structures of the isolated alpha and beta subunits of Na,K-ATPase were examined by CD spectroscopy in the dialysis buffer, which contained 0.01 or 0.02% SDS. All alpha subunits produced very similar CD spectra (Figure 2a), and consequently, the net secondary structures calculated from these data were all very similar (Table 1).

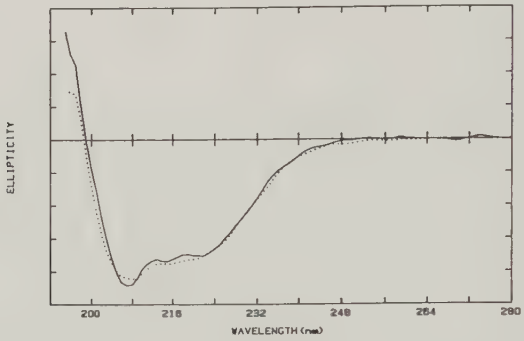

Figure 2a: Circular dichroism spectra of Na,K-ATPase alpha subunits isolated from dog kidney (————) and brine shrimp (••••••••).

This similarity suggests that in animal sources as phylogenetically diverse as mammals and brine shrimp, the structure of this vital enzyme is strongly conserved. The alpha subunits are composed of approximately 20% helix, 34% sheet, and 10% turn. If all the helix were in transmembrane segments, this would be enough to form $\sim$ 8-9 spans of the membrane. It is notable that in this environment the polypeptide does not fold in either of the ways which might have been predicted for a protein in the presence of SDS: either as a denatured random coil (caused by the repulsive interactions due to the charges on the bound detergent), or as a helical structure (promoted by the presence of the detergent which could stimulate the formation of intramolecular hydrogen bonds). Rather, it maintains a well-defined secondary structure. Furthermore, that structure is unlike the structure that would be predicted for this amino acid sequence by algorithms such as Chou and Fasman, (1979) or Garnier et al, (1978), which are empirically based on the folding of watersoluble proteins. However, the secondary

structure of the alpha subunit in SDS does seem to correlate with the number of hydrophobic (presumably helical) transmembrane segments predicted by hydropathy algorithms (Kyte & Doolittle, 1982). This is behavior typical of native membrane protein structures (Wallace et al, 1986).

The spectra of the beta subunits from different sources are also very similar, again indicating evolutionary conservation of net secondary structures (Figure 2b). In this case, the apparent secondary structures consist of 18% helix, 42% sheet, and 6% turn (Table 1).

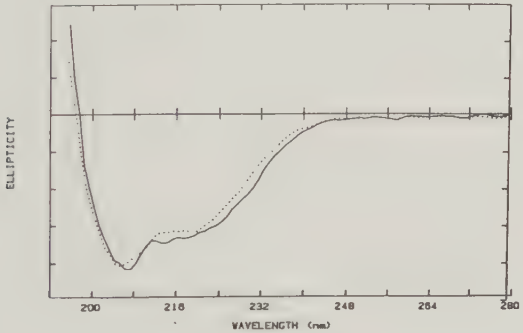

Figure 2b: Circular dichroism spectra of Na,K-ATPase beta subunits isolated from dog (————) and guinea pig kidneys (••••••••).

TABLE 1: Calculated Secondary Structures

Source	helix	structure	coil
		Alpha Subunit	
guinea pig	0.20	0.42	0.38
dog	0.19	0.44	0.37
brine shrimp 1	0.16	0.48	0.36
brine shrimp 2	0.18	0.48	0.34
		Beta Subunit	
guinea pig	0.18	0.48	0.33
dog	0.15	0.46	0.39
		Holoenzyme (Guinea Pig)	
Intact(E2 form)	0.20	0.42	0.37
"Synthethic"	0.20	0.43	0.36

Comparison to Holoenzyme:

To determine roughly how much refolding is involved when the alpha and beta subunits are associated, we can compare the sum of the alpha and beta subunit spectra (in the proper mass ratios) with the spectrum of highly purified holoenzyme in native membrane sheets (Gresalfi & Wallace, 1984) (Figure 3 and Table 1). Surprisingly, the sum of isolated alpha and beta in SDS is very similar to the holoenzyme, thereby suggesting not only that refolding does not involve a major net rearrangement of the secondary structures of the subunits, but that the subunits in SDS must retain a somewhat native-like structure, even in this "denaturing" detergent. Interestingly, the "synthetic" spectrum is most similar to that of the E2(K) form of the molecule which also seems to predominate in detergent solutions of the holoenzyme (DiNolfi & Wallace, 1986).

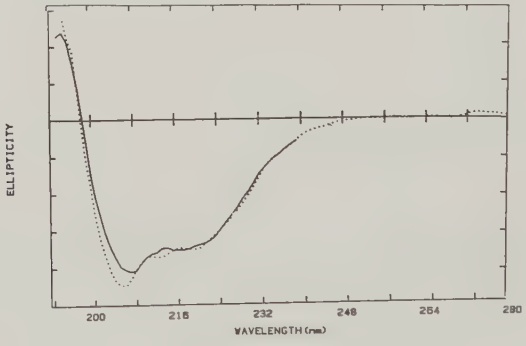

Figure 3: Circular dichroism spectrum of the E2 form of the purified holoenzyme from guinea pig (————) and the spectrum of a "synthetic enzyme" (i.e. the sum of the spectra of the isolated guinea pig alpha and beta subunits, weighted in the correct mass ratios) (••••••••••).

These comparisons suggest that recombination of the alpha and beta subunits may result in little net refolding of the molecules, although, of course, compensatory changes may occur. Neither of the isolated subunits exhibited any catalytic activity, however, so some unfolding must occur, but it may be localized to the region around the active site. Our future goals will be to physically recombine the alpha and beta and measure whether the activity of the enzyme can

be regained. It is expected that given the present results, this may not be as a difficult a task as originally anticipated.

ACKNOWLEDGEMENTS

We thank Dr. M. Gawinowicz for the amino acid analyses and Dr. I. S. Edelman for helpful discussions. The work was supported by NIH grants AM31089 and GM27292. B. A. Wallace was the recipient of a Camille and Henry Dreyfus Teacher Scholar Award during the course of these studies.

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AN INQUIRY INTO THE BINDING OF Cr-ATPa BY (Na^+ + K^+)-ATPase

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INTRODUCTION

The chromium (III) complex of 3'-O-{3-[(N-4-azido-2-nitrophenyl)amino]-3-tritiopropionyl}adenosine 5'-triphosphate (Cr-ATPa) is a photoaffinity reagent for (Na^+ + K^+)-ATPase (Munson, 1981). It can be inserted into the active site of (Na^+ + K^+)-ATPase while occupying the location to which the normal substrate binds during turnover (Munson, 1981; Kyte, 1981). It has also been reported that fluoresceinyl 5'-isothiocyanate (FITC) reacts specifically with the Lys-501 residue in the peptide HLLVMK(FITC)GAPER to inactivate (Na^+ + K^+)-ATPase. The similarity between the results obtained with FITC (Karlish, 1979) and Cr-ATPa suggested that these two disparate reagents nevertheless react with the same location in the sequence of the protein. If evidence that Cr-ATPa modifies the same lysine as that modified by FITC could have been obtained, the results from this experiment would have pinpointed the location of the modification.

An immunoadsorbent directed against the peptide HLLVMKGAPER has been used to single out the peptide containing the amino acid residue Lys-501 of the (Na^+ + K^+)-ATPase from tryptic digests. The results presented here show that Cr-ATPa does not modify Lys-501 of the α subunit. Hence, Cr-ATPa and FITC modify different residues in the protein. Sequence determination of the [^{3}H]-Cr-ATPa-labeled peptide is in progress.

METHOD

Native $(Na^+ + K^+)$-ATPase was isolated from canine kidney (Kyte, 1971). Purified $(Na^+ + K^+)$-ATPase was prepared by the method of Jorgensen (1974) and assayed for strophanthidin-sensitive $(Na^+ + K^+)$-ATPase activity (1100 µmol Pi/mg/hr)(Kyte, 1971). The peptide KGAPER was synthesized manually by solid-phase techniques (Stewart and Young, 1969) and was coupled to bovine serum albumin by the glutaraldehyde procedure (Walter et al, 1980). The resulting covalent complex was injected as an antigen into rabbits. Antibodies against the sequence GAPER were purified with an affinity column made from the synthetic peptide KGAPER and agarose beads by coupling with cyanogen bromide (March et al, 1974). These antibodies in turn were attached to agarose beads by cyanogen bromide activation to produce an immunoadsorbent. The capacity of the immunoadsorbent was determined by saturating it with synthetic peptide, eluting the bound peptide with acid, and determining by amino acid analysis the amount of peptide released. The capacity of the immunoadsorbent used for this experiment was 4.5 nmol of peptide (ml settled immunoadsorbent)-1. $[^3H]$-CrATPa was synthesized (Munson, 1983) and photolyzed in the presence of $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase was also labeled with FITC (Farley et al, 1984). Both samples were digested with trypsin (Farley et al, 1984). The supernatants resulting from centrifugation were passed over the immunoadsorbent.

RESULTS

The photolyzation of $[^3H]$-CrATPa in $(Na^+ + K^+)$-ATPase differs remarkably according to the presence or absence of ATP. Figure 1 shows that increase of tritium resulted only in the absence of ATP. When tryptic digests of the $(Na^+ + K^+)$-ATPase labeled with $[^3H]$-CrATPa were passed over a column of the immunoadsorbent directed against the carboxyl terminal sequence -GAPER and eluted with 0.1 M sodium phosphate at pH 2.5, Figure 2 (▲) shows that there is no tritium attached to the HLLVMKGAPER. In contrast, when the same amount of digest that had been labeled with FITC in the absence of ATP was passed over the same immunoadsorbent, a significant amount of HLLVMK(FITC)GAPER peptide was bound and eluted (see Figure 2, ●). This provides evidence that the binding site of $[^3H]$-CrATPa is not Lys-501. The determination of the composition of Cr-ATPa-labeled peptide is in progress.

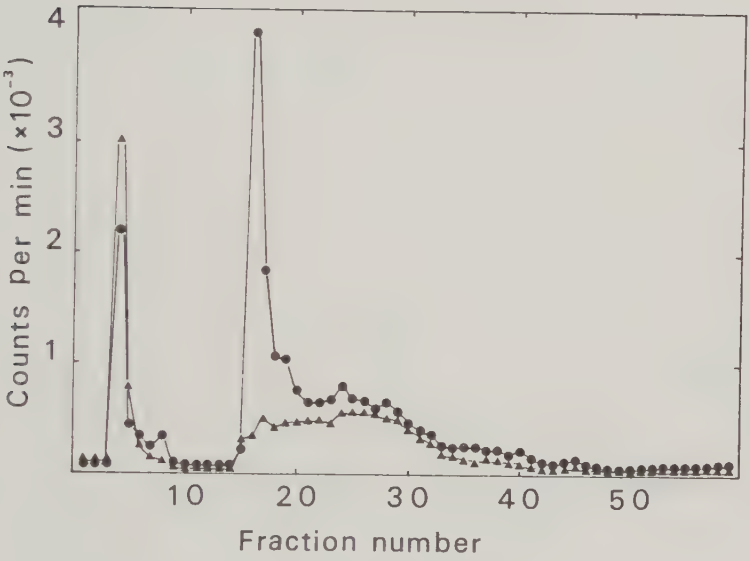

Figure 1. High pressure liquid chromatography of a tryptic peptide from [^{3}H]-CrATPa photolyzed in the presence or absence of ATP. ($Na^+ + K^+$)-ATPase (3 mg) and 40 nmol of [^{3}H]-CrATPa (2.2×10^{12} cpm μmol^{-1}) were present in 1 ml of buffer (1 mM EDTA, 25 mM imidazolium chloride, 250 mM sucrose) at pH 7.5. Samples were photolyzed for 10 min at 20°C in the presence and absence of ATP, respectively. They were dialyzed, washed again by ultracentrifugation, resuspended in 1 mM EDTA, 25 mM imidazolium chloride, pH 7.5, and digested with trypsin. The remaining particulate material was removed by ultracentrifugation; portions of the supernatants were lyophilized, redissolved in 0.1% trifluoroacetic acid, and injected onto a C_4-reversed-phase column that was then developed with a linear gradient of 0%-100% acetonitrile in 0.1% trifluoracetic acid delivered at 1 ml/min over 90 min. The fractions (1 ml) were collected, and their tritium content was determined by liquid scintillation. (▲) Enzyme photolyzed in the presence of ATP. (●) Enzyme photolyzed in the absence of ATP.

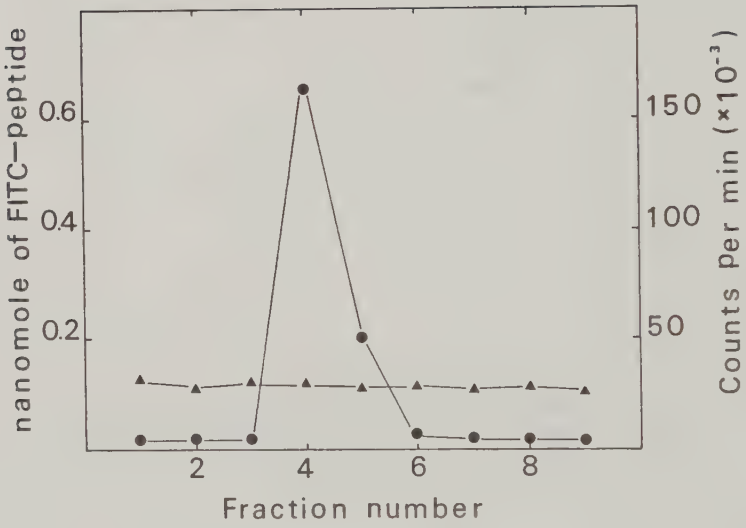

Figure 2. Immunoaffinity chromatography of tryptic digests of [^{3}H]-CrATPa-labeled (Na^+ + K^+)-ATPase and FITC-labeled (Na^+ + K^+)-ATPase on a column of immobilized anti-GAPER antibody. The supernatants of tryptic digests were passed directly over a column (2.5 ml) of agarose beads to which the anti-GAPER antibody had been attached (11 nmol capacity). The column was then washed with 15 ml phosphate-buffered saline and eluted with 0.1 M sodium phosphate at pH 2.5. The absorbance at 500 nm of each one ml fraction was determined, and the number of moles of labeled peptide HLLVMK(FITC)GAPER was calculated based on an extinction coefficient of 75,000. One ml fractions of [^{3}H]-CrATPa-labeled sample were submitted to liquid scintillation counting. (●) 3 nmol digest of (Na^+ + K^+)-ATPase labeled by FITC in the absence of ATP. (▲) 3 nmol digests of (Na^+ + K^+)-ATPase labeled by [^{3}H]-CrATPa in the absence of ATP. In all cases, the same relative amount of each digest based on the protein concentration of the original sample of (Na^+ + K^+)-ATPase was passed over the column.

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Oligomeric Structure: Physicochemical and Kinetic Evidence

Monomer - dimer.

One plus one makes two;
one can do,
so can two;
why two,
if one can do;
is the answer,
one and two.

J.C.S.

The Na^+,K^+-Pump, Part A: Molecular Aspects, pages 137-148
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OVERVIEW: THE OLIGOMERIC STRUCTURE OF THE NA,K PUMP PROTEIN

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INTRODUCTION

Throughout the history of protein chemistry molecular weights, subunit stoichiometry and mechanism have frequently been subjects of major controversy. However, the Na,K pump probably surpasses most other proteins in maintaining a long record of confusions and contradictions. Although the presence of two types of polypeptides, α and β, in the active protein complex is generally accepted, there is still uncertainty regarding (1) the function of the glycosylated β chain, (2) the number of subunits, substrate sites, and ion binding sites in each fully functional catalytic unit, and (3) the exact cyclic pathway followed in the ATP driven active transport of Na and K. Structural and functional models attempting to set aside these uncertainties have been proposed and often espoused with great zeal, but none are totally consistent with all the extant data.

It is apparent that proposed mechanisms for active ion transport in this and related systems depend upon the oligomeric state of the protein and upon possible changes in polypeptide association during the course of the catalytic cycle. The problem of the size of the catalytic unit, then, is central to all mechanistic discussion and must be addressed seriously and rigorously.

FACTS VS. FICTION

Eight years ago the subunit molecular weights for the

α and β polypeptides from shark rectal gland were determined rigorously using analytical ultracentrifugation (Hastings and Reynolds, 1979; Esmann et al., 1979). Two years later the size of these subunits from porcine kidney were reported (Freytag and Reynolds, 1981). We now have available the complete amino acid sequences for both the α and β chains from a number of different sources confirming the results from analytical centrifugation which provided an accuracy of 5 to 15% for the α subunit and 3 to 7% for the β subunit. (α=112,000 daltons, β=35,000 daltons).

With the aim of determining the minimal molecular weight of the functioning protein unit, two separate laboratories solubilized the Na,K pump protein from shark rectal gland in a large excess of non-ionic detergent. In the presence of dodecyl octaethylene monoether ($C_{12}E_8$) the mixed micelles were homogeneous and contained a protein with the subunit composition of $\alpha_2\beta_2$ (Esmann et al., 1979). However, when the pump protein was solubilized in hexadecyl eicosaethylene monoether ($C_{16}E_{20}$) the subunit composition was $\alpha_2\beta_4$ (Hastings and Reynolds, 1979). The enzymatic activity of both preparations was 9800 moles ATP hydrolyzed/mole protein-detergent complex/min, and since the molar turnover number determined in many different laboratories is 9000 moles ATP hydrolyzed/mole active site/min, one can readily calculate that these preparations contained 1 active site per 2 α chains. Does this mean that half the enzyme was inactive, or does it imply that the functional unit is a dimer? (It is worth noting that if half the enzyme had been inactivated, the inactivation did not occur as the result of solubilization since the total activity of the sample was unchanged on the addition of detergent.)

Since these data were published, several laboratories have demonstrated the existence of an active protein with the stoichiometry αβ in the presence of high concentrations of $C_{12}E_8$ (Brotherus et al., 1983; Esmann, 1984). It is now well established that a single protomer of both the Ca pump from the sarcoplasmic reticulum and the Na,K pump can assume all the identified intermediate states in the kinetic cycle (e.g. Martin, 1983; Martin and Tanford, 1984; Andersen, this volume). However, there are differences in the rate and equilibrium constants between detergent solubilized protomers and membrane-bound protein. Perhaps, more importantly, the detergent solubilized protomers that have been studied do not represent 100% of the original

membrane-bound pump protein - rather only 30-40% for the Ca pump and frequently considerably less than this for the Na,K pump.

Another method that has been invoked in an attempt to define the size of the minimal functional unit is radiation inactivation. Five reports of such experiments have appeared in the literature (Kempner et al., 1968; Ottolenghi et al., 1983a; Ottolenghi et al., 1983b; Karlish and Kempner, 1984; Jensen and Ottolenghi, 1985), and all but one find a target size for both the Na,K ATPase activity and the disappearance of the α subunit corresponding to an $\alpha_2\beta_x$ structure. This result suggests close proximity of the two catalytic subunits in the membrane but does not necessarily reflect specific functional interactions. It could equally well arise from a close packing density resulting from the high concentration of the pump protein within the lipid bilayer.

Attempts to determine the size of the minimal functional unit by counting substrate sites in purified, membrane-bound systems has led to utter confusion. It was pointed out at the previous International Meeting on the Na,K pump protein that the complacency of many years in accepting a 2 α chain structure was done away with by the report from Moczydlowski and Fortes (1981) that they were able to label more than one active site/2 α chains. There followed a scramble to demonstrate that all laboratories were overestimating protein concentration and hence underestimating the number of active sites. This hypothesis has proven to be incorrect (Jensen and Ottolenghi, 1983; Chetverin, 1986; Reynolds, unpublished data). Nevertheless, more than one group has reported greater than one active site per 2 α chains. Some particularly interesting findings related to this problem are presented in Table 1. Solubilization of purified, membrane-bound Na,K ATPase by increasing concentrations of $C_{12}E_8$ and subsequent determination of the number of ATP binding sites per mg protein shows that the preparation contains a mixture of competent and incompetent α chains. The apparent molecular weight inferred from ATP binding varies from one million to 172,000. Obviously, if one laboratory had chosen to solubilize this preparation at a detergent to protein ratio of 1.2, they would have concluded that the functional unit was $\alpha\beta_2$. On the other hand, if solubilization had been carried out at a ratio of 3, the functional unit would have been

identified as $\alpha_2\beta_2$.

TABLE 1. Solubilization of Purified Na,K Pump Protein

g $C_{12}E_8$/g protein	Apparent Molecular Weight[a]
0.375	10^6
0.6	256,000
0.9	208,333
1.2	172,414
1.8	217,391
3.0	243,900

a. Data from Jensen and Ottolenghi (1983). Apparent molecular weight of protein solubilized at the given ratio of detergent to protein is calculated from ATP binding capacity.

An additional set of experiments relating to subunit stoichiometry should also be considered. A quantitative determination of n-terminal groups has been reported (Chetverin, 1986) using purified, membrane-bound Na,K pump protein with a specific activity of 30 IU and a reported purity of 90-95%. The ratio of β polypeptide n-terminals to those of the α polypeptide was 2 - not 1 as would be expected if the basic protomeric unit is αβ. The author proposed that 50% of the α subunits contained blocked amino terminals. However, if one calculates from the published amino acid sequence the amount of protein present as α and β polypeptides assuming equal molar ratios of the two subunits, one obtains 1.1 to 1.35 mg αβ per mg of total protein in the sample. Do these results mean that the correct subunit stoichiometry is $\alpha\beta_2$? If the author's hypothesis regarding exactly 50% blocked n-terminal α polypeptides is correct, does this finding relate to the 50% α subunits that are incompetent in ATP binding experiments as reported by Jensen and Ottolenghi?

POSTULATED PREPARATIVE PROBLEMS

Intact microsomal preparations containing the Na,K pump cannot be studied directly, but must be made "leaky"

by some means in order to provide access of solvent to both sides of the membrane. By far the most popular technique is that developed by Skou and later Jorgensen which involves titration of the microsomes either with DOC or SDS. The partitioning of both these detergents into the bilayer leads to fragmentation of the membranes, and further purification is then accomplished by separation of fragments enriched in the Na,K pump protein on density gradients.

Figure 1 shows three SDS titrations of microsomes prepared from porcine kidney. It is apparent that (1) considerable variation occurs in the original microsome preparation and (2) that a very narrow concentration region exists where maximal activity occurs. This type of result raises the possibility of a denaturation phenomenon superimposed upon the fragmentation process. The use of SDS poses a particularly serious problem because of its ability to denature the water-soluble domains of membrane proteins and because of its slow rate of flip-flop across phospholipid bilayers. This latter property imposes a kinetic barrier to the attainment of equilibrium and can result in a lack of reproducibility among experiments such as those shown in Figure 1. It is unfortunate that data documenting the fragmentation of microsomal preparations and the subsequent appearance of ATP hydrolytic activity are rarely published in studies attempting to elucidate the structure and mechanism of the Na,K pump protein.

In contrast to the narrow detergent concentration range of maximal activity shown in Figure 1, Figure 2 shows the large plateau region observed when a long alkyl chain, non-ionic detergent is used for membrane fragmentation. In this case partitioning into the membrane is also an extremely slow process requiring hours at room temperature (Reynolds et al., 1985).

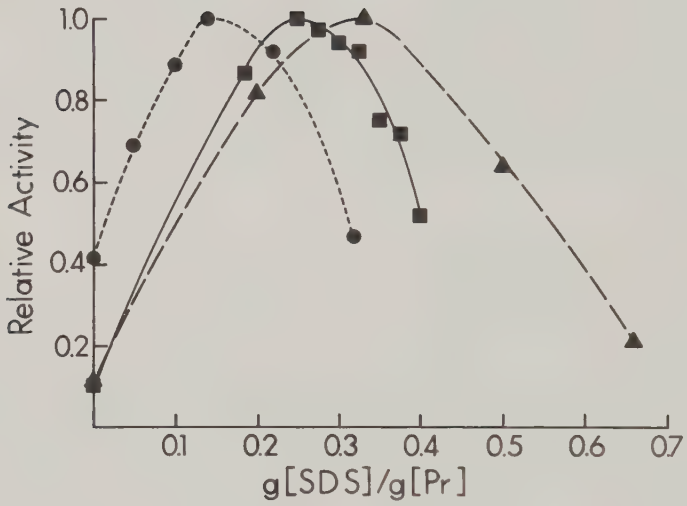

Figure 1. Titration of porcine kidney microsomes with sodium dodecylsulfate at room temperature. ● Data from Jensen et al., 1984, -incubation time 24 hrs. ■▲ Unpublished data from Reynolds - incubation time 60 min.

The process of detergent opening of sealed microsomes is not the only source of potential problems. When the SDS titrated material is further purified by density gradient centrifugation, the normal recovery of Na,K ATPase activity is usually considerably less than 100%. Table 2 presents one published example. Since it is relatively rare for scientific papers in this field to provide this type of information, one is frequently in the position of being unable to evaluate how representative scientific findings are of the true in vivo structure and function of this transport protein.

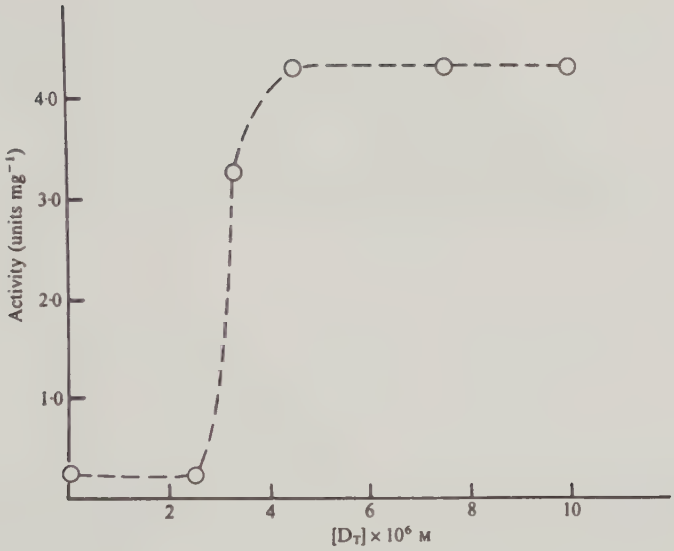

Figure 2. Appearance of ouabain sensitive Na,K ATPase activity on the addition of Brij 58 to porcine kidney microsomes at 37°. Membrane lipid concentration=1.5×10^{-6}.

TABLE 2. Recovery of Protein and Enzymatic Activity From SDS Treated Microsomal Preparations.[a]

	Total Ouabain Sensitive ATPase Activity	Total Protein
Microsomes + SDS	138 IU	10.22 mg
Purified Membrane Fragments	71 IU	2.84 mg

[a]From Hardwicke and Freytag, 1981

It should also be kept in mind that further treatment of these SDS treated and purified membrane fragments with $C_{12}E_8$ to obtain protomeric forms of the Na,K pump protein yields a maximum of 70-80% solubilized protein (e.g. Jensen and Ottolenghi, 1983). Therefore, if the material described in Table 2 had been dissolved in excess $C_{12}E_8$ to obtain the protomeric form, the final sample would represent a maximum of 41% of the original membrane-bound protein.

SUBUNIT INTERACTIONS

Let us turn now to the subject of subunit interactions - granting that all the evidence to date points at the very least to interactions between α and β - and perhaps to interactions between protomers of $\alpha\beta$.

Table 3 lists the forces that occur in aqueous and hydrophobic solvents. In principle all of these forces can contribute to protein-protein association in aqueous solution. For example, the removal of hydrophobic amino acid residues from contact with water by formation of an interface between two polypeptides contributes favorably to the free energy of association, and one might expect that this would be the overwhelming contribution to self-association of the extra-bilayer domains of an intrinsic membrane protein as it is to the oligomerization of many water-soluble proteins.

In considering the interaction between those portions of proteins that are immersed in the hydrophobic interior of the bilayer, however, hydrophobic forces are no longer extant. Self-association in this milieu, then, presents some intriguing problems. If it occurs at all, association must be governed by one or more of the following: (1) dispersion and/or dipolar interactions between the membrane-embedded domains are stronger than the same interactions between the protein and the hydrocarbon tails of the lipids; (2) individual charges of opposite sign occur in the membrane-embedded domains in an appropriate configuration to facilitate ion-pair formation; (3) association occurs by virtue of hydrogen bond formation between adjacent protein domains.

TABLE 3. Intermolecular Forces

Within the Bilayer	In the Aqueous Milieu
Dispersion Forces	Dispersion Forces
Dipole Interactions	Dipole Interactions
Hydrogen Bonds	Hydrogen Bonds
	Hydration Forces
Chelation	Chelation
Ionic Forces	Ionic Forces
	Hydrophobic Forces

How strong must specific forces be to promote association of membrane bound proteins? We frequently ignore the enormous difference in concentration between polypeptides restricted to the volume of a lipid bilayer and those dissolved in 55.5M water. Table 4 presents the free energy required for 99% dimerization of a 100,000 dalton protein associated with a biological membrane at two different weight ratios of protein to lipid. Note the large difference in free energy required for self-association in this system as compared to that for a normal water-soluble oligomeric structure - a difference that simply reflects the higher local concentration within the membrane.

TABLE 4. Free Energies in Mole Fraction Units of Dimerization of a 100,000 Dalton Polypeptide

Weight Fraction protein/lipid	Mole Fraction	Free Energy kcal
0.75	0.023	-7.7
0.50	0.008	-8.2

The free energy of oligomerization of multi-subunit water-soluble proteins is -14 to -20 kcal in mole fraction units.

It is instructive to consider that the dipolar interaction between 2 - 25 residue - antiparallel helices embedded in a medium of low dielectric constant is estimated to be approximately 2-3 kcal, and, therefore, four such group interactions would provide sufficient free energy in systems such as membrane-bound, kidney Na,K pump for oligomerization. On the other hand, the concentration of the pump in erythrocytes is orders of magnitude lower, and much higher free energies would be required for self-association.

One can disrupt or promote subunit interactions by changing the solvent milieu. In the case of membrane bound proteins with both a hydrophobic and a hydrophilic domain, both solvents must be considered. We know that detergents with small hydrophobic domains (e.g. octyl glucoside, $C_{12}E_8$) will dissociate water-soluble proteins by competing with subunit interactions at hydrophobic interfaces, and it would be expected that the same phenomenon might occur with the water-soluble domains of the pump proteins. There are also extensive studies by Timasheff and coworkers on the effects of alterations in preferential hydration on self-association of proteins. This latter work should engender some concern with respect to the widespread use of high concentrations of sucrose and glycerol with preparations of the Na,K pump protein. In general, it would seem prudent to maintain all solvent systems as close to those encountered in vivo as possible if we are to correctly interpret experimental observations.

SUMMARY

The purpose of this paper has not been to espouse a particular structural model for the Na,K pump protein. Rather an attempt has been made to produce an awareness of a body of scientific literature that raises some serious questions and to suggest that complacency on the subject of the oligomeric vs protomeric state of this (and perhaps other) transport proteins is inappropriate.

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OVERVIEW:

LIGAND BINDING SITES OF $(Na^+ + K^+)$-ATPase: NUCLEOTIDES AND CATIONS

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INTRODUCTION

I was asked by the conference organizers to present a critical overview of the above topic, with emphasis on the unresolved problems. I shall consider the nucleotide binding sites and the related cases of the monovalent cation binding sites. As interesting and important as they are, the divalent cation sites will not be covered here.

A casual examination of the recent literature of the field would show that when the all-important issue of the reaction mechanism of the pump is considered, the most commonly made assumptions about the ligand sites are that the enzyme has (a) a single nucleotide site with different affinities on the two principal conformations of the enzyme; and (b) a single monovalent cation binding center whose specificity and affinity alternate between the two principal conformations. A less casual examination of the literature would show, however, that the major problems of the field over the past two decades have involved disagreements on the validities of these assumptions concerning the ligand sites. To address these issues properly, I believe it is necessary that we clarify some misunderstandings; and to do this, we must begin with a brief history of the events that have brought us to where we are today.

The early studies showing the nonhyperbolic and negatively cooperative nature of the substrate-velocity curve of the enzyme, led to the suggestion of the existence of a mixture of unidentical enzymes (Czerwinski *et al.*,

1967; Neufeld and Levy, 1969). Without the assumption of multiple enzymes, however, Skou (1971) suggested that ATP must have two effects on the enzyme; one being different from its participation in enzyme phosphorylation. The work of Post's laboratory (Hegyvary and Post, 1971; Post et al., 1972) led to the proposal that this additional effect of ATP was due to its binding with low affinity to E_2K^+, increasing the rate of release of the newly proposed occluded K^+ from this enzyme species. Post et al. (1972) realized that within the reaction scheme of the enzyme (the one we now know as the Albers-Post cycle) the two effects of ATP could be either at the same binding site on two different forms of the enzyme, or at two distinct sites: a high-affinity catalytic site and a low-affinity regulatory site. Soon after this, the possibility of the functional significance of the oligomeric structure of the enzyme was suggested independently by two laboratories (Repke and Schon, 1973; Stein et al., 1973); leading to the notion that the Albers-Post cycle could proceed out of phase in the two halves of a dimer, thus allowing the single ATP site of each protomer to function alternatively as the catalytic and the regulatory sites for the functional dimer. This pushed aside the consideration of distinct catalytic and regulatory sites for ATP, and from here on the issue of the multiplicity of ATP sites became intertwined with that of the oligomeric structure of the enzyme. Although there were differences in the versions of alternating site mechanism proposed subsequently, by the end of the decade it was widely accepted that the dimeric enzyme operating through such a mechanism could bind Na^+ and K^+ simultaneously, was half-site reactive in respect to phosphoenzyme formation, could bind ATP to its phosphorylated form, and could not function as a coupled pump unless it were a dimer. While at the time each of the above premises had a reasonable basis in facts, soon data in conflict with some began to appear. Unfortunately, with these developments it also became evident that the notoriety of alternating site mechanisms had created a surprising misunderstanding in the field; namely, the assumption by many that the validity of each feature of the above-described dimer depended on the validity of all of its presumed properties. And it is this misunderstanding that has caused much confusion in the debates concerning the ligand binding sites of the enzyme. It is disheartening to see, for example, that to this day data that presumably rule out half-site reactivity in respect to phosphoenzyme formation are taken to mean that the existence of an oligomer has been ruled out; as if every oligomer with

negative site-site interactions must exhibit half-site reactivity. The consequence of such misunderstandings has been the bizarre polarization seeming to indicate that one must believe either in the oligomeric enzyme with an alternating site mechanism and the features indicated above, or in an enzyme that has none of the above features, and whose monomer with a single ATP site and a single cation binding center is the fully competent unit of the pump. The simple truth is that most of the available evidence supports neither of the two extremes.

Trying to avoid the confusion created by the above misunderstandings, in the following sections I shall first summarize the different lines of evidence that are pertinent to the issue of the number of the ligand binding sites, focussing on these questions: Does the enzyme have one or more ATP sites? Does it have one or more sets of monovalent cation sites? It will be noted that without getting involved in the monomer-dimer debate, and without considering the merits of an alternating site mechanism, the answers are favorable to the multiplicities of the ligand sites. I shall then consider the possible origins of these multiplicities, and some of their implications for the enzyme's function.

MULTIPLICITIES OF THE ATP SITES

Some of the points addressed below, have been discussed in more detail elsewhere (Askari, 1987).

Half-site Reactivity of the Phosphorylation Sites.

The assumption that only half of the α-subunits are phosphorylated, and the data supporting this assumption, were integral parts of the alternating site mechanisms. A few years ago, we were led by some colleagues to believe that if the true protein content of a preparation were determined accurately by quantitative amino acid analysis, it could be shown that all potential phosphorylation sites are indeed phosphorylated (e.g., Craig and Kyte, 1980). However, based on more recent work (Chetverin, 1986; Ottolenghi and Jensen, 1985), we can no longer be certain of the reliability of the amino acid analysis, and the inferiority of the Lowry method, as applied to the membrane-bound preparations of the enzyme. This, and the impurity of such preparations, make it doubtful that the

question of whether or not the enzyme may exhibit half-site reactivity can be settled by experiments that rely on the determination of the absolute concentration of the enzyme. On the other hand, experimental approaches that do not rely on the knowledge of "true" protein content, indeed do show ligand-induced half-site reactivity of the phosphorylation sites (Askari et al., 1983). But these findings, and similar data showing half-site reactivities of other ligand binding sites (Askari, 1987), tell us little about the monomer-dimer issue or the reaction mechanism; they simply show the existence of two equal populations of unidentical sites, or negative site-site interactions between two equal populations of identical sites.

Negative Cooperativities of the Equilibrium Binding Plots.

The meanings of such plots, either for high-affinity ATP binding (e.g., Ottolenghi and Jensen, 1983) or for phosphoenzyme formation from Pi (Askari and Huang, 1984), are clear: The enzyme must have multiple ATP (or Pi) sites with induced or initial asymmetry. The number of sites can not be determined from these plots alone; but taken together with half-site reactivity data, we must conclude that we are dealing with two equal populations of sites. To reiterate, in principle, there is no reason why such a dimer of sites could not be on the same polypeptide chain.

Ratio of Nucleotide Binding Sites to Phosphorylation or Ouabain Binding Sites.

In highly purified and non-vesicular preparations of the membrane-bound enzyme, the maximal number of high-affinity ATP binding sites is equal to the maximal phosphorylation capacity or the ouabain binding capacity of the enzyme. The long-standing suggestions that there may be ATP binding sites on the enzyme in addition to the easily detectable high-affinity ATP sites, are now confirmed by the unequivocal demonstration of two co-existing ATP sites per ouabain binding site (Askari, 1987; Kakar et al., 1985). Note that the two populations of sites identified by the equilibrium binding plots (the previous section) are within one of the two sets of ATP sites identified here.

ATP Binding to the Phosphoenzyme and the Enzyme-Vanadate Complex.

It is now well-established that ATP may bind to the phosphoenzyme and the enzyme-vanadate complex (Askari, 1987; Forbush, 1987b). Taken alone, this does not prove the multiplicity of ATP binding sites. However, since the existence of two ATP sites per phosphorylation site, and the simultaneous occupation of both by ATP, have been established (the previous section), the most straightforward explanation is that ATP phosphorylates at the first set of sites, and may bind to the other regardless of whether the first site is phosphorylated or occupied by intact ATP.

Phosphorylation by ATP of the Enzyme That Has One of Its Nucleotide Sites Occupied.

Several amphiphilic compounds, including liponucleotides, have the same effect on the enzyme activity as the low-affinity effect of ATP within the Albers-Post cycle (Huang et al., this conference and references therein). Since phosphorylation by ATP proceeds normally in the presence of these regulators, one is tempted to conclude that while these nucleotide substitutes are occupying the non-phosphorylatable ATP site, phosphorylation at the high-affinity ATP site is occurring. This argument would be faulty, however, since these regulators bind to the enzyme reversibly, and it is possible that they are no longer on the enzyme when ATP begins to phosphorylate. Experiments of Scheiner-Bobis et al. (1987; and this conference) are more decisive. They show that cobalt-tetramine-ATP which interacts with the low-affinity ATP site irreversibly, also leaves enzyme phosphorylation by ATP intact; indicating the multiplicity of the nucleotide binding sites.

Negative Cooperativity of the Substrate-Velocity Curve.

Taken alone, the nature of this curve tells us nothing about the number of ATP sites, the monomer-dimer issue, or the reaction mechanism of the enzyme (Askari and Huang, 1985; Rossi and Garrahan, 1985). However, considering that multiplicity of ATP sites has been indicated by the evidence discussed above, it would be surprising if the multiple sites were not responsible, at least in part, for the cooperativity of this plot.

MULTIPLICITY OF THE CATION SITES

Questions concerning the number of cation sites include the following: How many Na^+ or K^+ ions bind to the transport sites of the functional unit of the enzyme? Are there additional non-transport sites of functional significance? Are there separate sites for Na^+ and K^+; and may Na^+ and K^+ bind to the enzyme simultaneously? In principle, these questions are not different from those raised about the multiplicities of the other substrate (ATP) and the other product (Pi) of the enzyme; and just as in the case of the nucleotide sites, we should try to see if these questions can be answered without the interference of the controversies on the quaternary structure and the reaction mechanism of the enzyme. (Before we get on with this task, it is appropriate to note that questions on the sidedness of the Na^+ and K^+ sites are entirely separate from those on numbers, and will not be addressed here.)

Cooperativities of the Cation-Velocity Curves.

The nonhyperbolic nature of these plots observed in a multitude of studies, either on ATPase activity and other partial reactions of the enzyme, or on ion fluxes, are again of little value in establishing the multiplicities of Na^+ and K^+ sites. In principle, just as in the case of the ATP-velocity curve, any nonhyperbolic curve for Na^+ and K^+ could be either due to multiple sites, or because of hysteresis or other so-called steady-state effects in an enzyme with only one site, or due to a combination of these. [It is also true, however, that regardless of the number of sites, the question of whether or not Na^+ and K^+ are mutually exclusive, should be amenable to testing by such kinetic experiments. The fact that more than two decades of often elegant kinetic studies have had less than spectacular success in resolving this seemingly simple question, is another testimonial to the complexity of the pump and to the futility of attempts to describe it through simple models (Askari, 1987).]

Equilibrium Binding Plots of Cations: Cooperativities and Stoichiometries.

The pertinent studies have been cited and discussed by Glynn (1985) and Jensen *et al.* (1984). The conclusions to be drawn from these are as follows: 1. In different

laboratories, 2-5 K^+ (or Rb^+) binding sites of high affinity per phosphorylation site (or ouabain site) have been detected. If we take the lower limit of this stoichiometry, the widely held assumption that for either Na^+ or K^+ there are more than one high-affinity transport sites per α,β-protomer should be questioned; since as discussed above, we can no longer be certain that in membrane-bound preparations every α-subunit is indeed phosphorylated. In the case of Na^+, however, 3 Na^+ sites per phosphorylation site have been detected consistently. Needless to say, this value and the values of higher than 2 found in some studies on K^+, would clearly require the existence of more than one site per α-subunit regardless of whether the enzyme is half-phosphorylated or fully phosphorylated. 2. In most studies on the native enzyme, the equilibrium binding plots for Na^+ and K^+ (or Rb^+) have been found to be positively cooperative. These clearly support the existence of multiple interacting high-affinity sites. 3. The possibility of the simultaneous bindings of Na^+ and K^+ have been tested in two studies. Yamaguchi and Tonomura (1980) have noted that 3 Na^+ and 2 K^+ may bind simultaneously, but Matsui and Homareda (1982) have found that the two ions are mutually exclusive. The possible technical shortcomings of the former study have been discussed in the latter. The conclusions of Matsui and Homareda (1982), however, should also be viewed with caution, since they used ouabain sensitivity as the criterion for identification of high-affinity cation sites, and we now know that some high-affinity cation binding to the ouabain-complexed enzyme does occur (See below and Kakar *et al.*, this conference).

Nonequilibrium Binding Data.

In equilibrium binding studies, a major problem is to assure that filtration or centrifugation techniques do not disturb equilibrium between the bound and unbound ligands significantly. On the other hand, where occluded ions are measured, care must be exercised to assure that measurements are made at a time when the bound ion is not in equilibrium with the unbound ion. One technique's fault is the other's virtue. The stoichiometry of occluded K^+ (or Rb^+) is a minimum of 2 per phosphorylation or ouabain site (Glynn and Richards, 1982; Esmann, 1985; Forbush, 1987a); and that of occluded Na^+ is a minimum of 3 per phosphorylation site (Glynn *et al.*, 1984; Esmann and Skou, 1985). The approximate equalities of these stoichiometries with those of most equilibrium binding studies suggest one

of two alternatives: 1. In the absence of deoccluding agents, all ions that are bound to high-affinity sites are occluded. 2. The equilibrium binding studies have been faulty in design; thus measuring only the occluded ions and allowing some high-affinity binding site to go undetected.

Regardless of the values of stoichiometries, experiments on the deocclusion rates clearly indicate that the total pool of the occluded K^+ or Rb^+ consists of two equal but unidentical populations (Glynn, this conference; Glynn et al., 1985; Forbush, 1987b and this conference).

Characterization of Cation Sites Through Nucleotide Binding Studies.

Jensen et al. (1984) have used the effects of Na^+ and K^+ on the equilibrium binding of labeled ADP to the enzyme in order to identify the binding sites for the cations. That such an approach may easily provide a reliable estimate of the affinities of the cation binding sites could have been predicted. These investigators determined not only the affinities, however, but through an elegant analysis of the resulting cooperative binding plots, they concluded that there were 5 high-affinity cation binding sites (occupied by either K^+ or Na^+ in a mutually exclusive manner) per total pool of high-affinity ATP sites. (Recall that this ATP pool, consisting of two equal populations of ATP sites, is equal to the maximal phosphorylation capacity of the enzyme.) Although the complexities of the analyses used suggest that the conclusions should be viewed cautiously, the agreement of the stoichiometry with those obtained in some of the direct equilibrium binding studies is intriguing.

Cation Binding Sites of the Ouabain-Complexed Enzyme.

The affinities, and possibly the numbers, of the cation binding sites of the complex of the enzyme with any ligand may be identified by studying the effects of cations on the rate of release of the ligand from the enzyme. Through this strategy, limited information had been obtained over the years about some Na^+ and K^+ binding sites of the phosphorylated enzyme (Hara and Nakao, 1981; Norby et al., 1983; Yoda and Yoda, 1986), the vanadate-complexed enzyme (Huang and Askari, 1984), the enzyme-ouabain complex (Akera et al., 1974; Lee and Fortes, 1985), and the complex

of the enzyme with occluded K^+ (Forbush, 1987a). Recently, we made a systematic study of the monovalent cation binding sites of the ouabain-complexed enzyme through this technique. The results of these studies are summarized elsewhere in this conference (Kakar et al.). Some of the pertinent conclusions are as follows: 1. The ouabain-complexed enzyme has mutually exclusive Na^+ and K^+ binding sites with high affinities (0.1-1 mM) similar to those of the transport sites of the native enzyme. 2. The complex has sets of sites with low affinities (0.2-0.6 M) that can be occupied simultaneously by Na^+ and K^+. ATP is an allosteric regulator of the low-affinity set of the positively cooperative Na^+ sites. 3. The sets of low-affinity and high-affinity cation sites can also be occupied simultaneously. The functional significance of the low-affinity cation sites is clearly indicated by their interactions with the ouabain binding sites and the ATP sites. Whether these low-affinity sites are allosteric sites as suggested by others (Karlish and Stein, 1985; Robinson this conference), or access channels as postulated for other transporters (Tanford, 1983, 1985), or discharge sites as usually assumed (e.g., Hara and Nakao, 1981), their coexistence with the high-affinity sites, and their simultaneous occupancies by Na^+ and K^+, show that the functional unit of the enzyme has multiple sets of cation binding sites.

ORIGINS OF THE MULTIPLICITIES OF THE LIGAND SITES

The evidence I have summarized above clearly indicates that the functional unit of the enzyme has multiple nucleotide binding sites and multiple sets of Na^+ and K^+ binding sites. There is also new evidence (Kakar et al., this conference) to support the old notion that there may be multiple ouabain binding sites on the enzyme. The first question to be raised is whether or not this multiplicity can be accommodated within the structure of an α-chain (or an α,β-protomer). The available structural data (reviewed elsewhere in this conference) providing important but limited information about the high-affinity ATP site, the phosphorylation site, and the ouabain site are not sufficient to answer the above question. While we wait for the expected rapid progress of the current structural studies, however, we should keep in mind that there is no compelling reason against the possibility of the existence of multiple sets of several ligand sites within a single chain. The next logical questions are the following: Since most

enzymes and membrane proteins function as oligomers and multicomponent assemblies, is it possible that the noted multiplicity of sites is due, partly or entirely, to the existence of interacting protomers within the functional unit of the enzyme? Is there evidence, independent of the multiplicity of sites, that indicates the presence of interacting protomers? The subject of the oligomeric structure of the enzyme is reviewed separately in this conference; therefore, I shall only emphasize two points: First, since an evolutionary relationship between $(Na^+ + K^+)$-ATPase and other transport ATPases has been clearly demonstrated, and since there is good genetic evidence for the oligomeric structure of one of these; i.e., the Kdp system (Epstein, 1985), it is more than likely that all of these systems operate as oligomers. Second, as reviewed in detail elsewhere (Askari, 1987), chemical cross-linking experiments have clearly shown the presence of contact areas between two α,β-protomers, and have established that such contact is ligand-regulated and not due to random collisions of these protomers within the membrane. There is little ambiguity in the meaning of these findings: The membrane-bound enzyme is indeed an oligomer consisting of multiple α,β-protomers. In view of the above, it would be silly not to consider the interprotomer site-site interactions as the origin of some noted multiplicities such as the ligand-induced cooperativities of the phosphorylation or the ATP binding plots. As discussed before (Askari, 1987), however, the occurrence of interprotomer site-site interactions does not rule out other origins of multiple nucleotide sites; and it remains to be seen whether or not the ATP sites that are in excess of the phosphorylation sites are distinct regulatory sites that coexist with the high-affinity sites on the same protomer. The situation is not much different in regard to the cation sites. While the best available data indicate the presence of more than one binding site per protomer, it would be a mistake to take this intraprotomer multiplicity as evidence against the existence and the functional significance of interprotomer interactions among the cation sites.

When oligomeric structure is a source of functional multiplicity of ligand sites, the possible dissimilarity of protomers must be considered. Years ago this issue was the subject of many experiments and debates in the case of glyceraldehyde-3-phosphate dehydrogenase (e.g. McQuarrie and Bernhard, 1971). That the protomers of $(Na^+ + K^+)$-ATPase may be slightly dissimilar was suggested by Repke and Dittrich (1979). This possibility was not supported by

studies of Ottolenghi and Jensen (1983) indicating that the negative cooperativity of the equilibrium binding plot for ATP was due to ligand-induced asymmetry in an oligomer of identical protomers. On the other hand our studies suggesting the existence of dissimilar but mutually exclusive ouabain binding sites (Kakar et al., this conference) are most easily accommodated by an oligomer of dissimilar but interacting protomers. It is important to note that, in principle, dissimilar protomers showing preexisting asymmetry in respect to one ligand, may exhibit perfect symmetry in respect to another ligand; and that the existence of such quasi-identical protomers in membrane proteins is now well-established (e.g., Ratnam et al., 1986 and references therein). How realistic is the possibility of such a hybrid oligomer in the case of $(Na^+ + K^+)$-ATPase? The existence of several isoforms of the α-subunit, and the potential for the occurrence of several such forms for the β-subunit, are now well-established (this conference). If two isoforms of either subunit exist in the same cell, it would be reasonable to postulate the existence of a hybrid oligomer with dissimilar protomers. Even in a cell that contains a single isoform of each subunit, however, a hybrid oligomer could consist of post-translationally modified and unmodified protomers. This possibility is in fact supported by the recent findings of Chetverin (1986). It is of interest to note that the dissimilarity, or the quasi-identity, of the two α-subunits of the nicotinic receptor also seems to be due to post-translational modification of otherwise identical chains (Ratnam et al., 1986).

IMPLICATIONS FOR THE ENZYME'S REACTION MECHANISM AND FUNCTION

Although consideration of the reaction mechanism is not within the scope of this presentation, I want to close by touching upon several functional implications of the multiplicities of the ligand sites.

1. In the beginning, I expressed the opinion that the available evidence supported neither a monomeric single-site enzyme, nor an oligomeric enzyme with an alternating site mechanism. If my interpretations of the data are correct, as summarized above, it should be evident to the reader why the monomeric single-site enzyme is not tenable. But if the enzyme is indeed an oligomer with multiplicity of sites as I have indicated, why can it not fit into an

alternating site model? Perhaps it can. The problem is, however, that the proposed alternating site models have been more in the nature of cartoons rather than reaction mechanisms that can be subjected to quantitative tests. This is not to pick on the cartoonists, but to point out the fact that until recently there have been few attempts in all of enzymology to incorporate multiple sites and site-site interactions into the steady-state properties of the reaction mechanisms. We are fortunate that Plesner (1987) has pioneered this approach in our field. Note that Plesner's model is not the alternating site scheme, but his approach which is based on the formalism of Hill (1975) for enzyme-enzyme interactions should be applicable not only to other dimeric models, but also to monomers with multiple interacting sites (see below).

2. Since it seems fairly certain now that at least in the case of cation sites we are dealing with multiple sites within a protomer, we may ask whether there are any functional difference between interprotomer and intraprotomer multiplicities of sites. In principle, the energetics of cooperativities between two ligand sites are the same regardless of whether the two are on the same polypeptide chain or on two subunits of an oligomer (Weber, 1975). Hence, in some respects it makes little difference whether the two interacting K^+ sites (Forbush, 1987b and this conference; Glynn, this conference; Glynn *et al.*, 1985) are on a monomer or a dimer; if we are going to incorporate the two sites of a monomer into the reaction mechanism of the pump, the issues to be considered are the same as those that have been addressed for many years in the case of oligomeric proteins and enzymes (e.g., may the two sites change 'conformation' in sequence, or must they do so in concert?) On the other hand, since it is generally the case that the bonds linking the polypeptide chains are weaker at the boundaries between protein subunits than those within a globular protein, the interprotomer multiplicities may be more convenient targets for control. It is for this reason that when a number of site-site interactions for several ligands are disrupted by a regulator such as ouabain (Kakar *et al.*, this conference), it is reasonable to suspect that control is being exerted at the interprotomer domain; i.e., the common boundary for several site-site interaction.

3. Granted that the native enzyme is an oligomer with both intra- and intersubunit site-site interactions, what functional significance could the latter have if the

monomeric enzyme is also shown to be active? The most serious of the red herrings that have come out of the monomer-dimer debate is the one about the detergent-solubilized monomer that is claimed to have "complete function". I have discussed in some detail (Askari, 1987) why I believe that a catalytically competent monomer of this enzyme has not been demonstrated to date; but that is not what I want to repeat here. Let us assume that future studies (or perhaps those presented at this conference) establish that a free-standing protomer does show $Na^+ + K^+$-ATPase activity. Would this mean, as many have already claimed it does, that the monomer is the functional unit of the pump and that the oligomeric nature of the enzyme is irrelevant to function? Of course not. It is true that some oligomeric enzymes need to remain oligomers if they are to display activity (perhaps because the active site spans two monomers, or because the monomer is simply unstable), and that in some specific models the oligomeric state improves catalytic efficiency (e.g., Ricard and Noat, 1986; Plesner, 1987). This should not imply, however, that catalytic incompetence of the monomer is a necessary feature of all oligomeric enzymes, and that the only "purpose" of the oligomeric structure is to enhance activity. It seems to have been forgotten that theories of cooperativities of oligomeric proteins were developed not to explain function, but control of function; and that the possibility was in fact considered that the loss of catalytic efficiency may be a price that an oligomer must pay in order to gain the capacity for control (Monod *et al.*, 1965). We now know, of course, that being oligomeric is a common way, but not the only way, through which a protein gains this capacity.

Considering the above, it should be clear that the precise role of the interprotomer site-site interactions of this enzyme will not be known until we have an extensive comparison of the properties of the native enzyme and a free-standing monomer that remains a monomer. Because of the complex non-solubilizing effects of detergents on the enzyme (Huang *et al.*, 1985), it is doubtful that such a comparison would be possible by the use of detergent - solubilized preparations. Perhaps it can be done, however, through protein engineering as it has been attempted for other enzymes (e.g., Jones *et al.*, 1985).

4. The shape of the substrate-velocity curve of this enzyme, and its K_m value, started the questions about the multiplicities of the ligand sites. We have been so pre-

occupied with mechanisms that on the whole we have neglected the potential significance of the phenomenon to the control of function. It is often said that intracellular ATP level (say 2-5 mM) is so much higher than the K_m of the pump for ATP (say 0.2-0.5 mM) that the cooperativity of the substrate-velocity curve can not be of any practical value to the enzyme. With physiological levels of intracellular K^+, however, the K_m for ATP may be much closer to intracellular concentrations of ATP (Kakar et al., 1987); and under these conditions the subsensitivity of the negatively cooperative substrate-velocity curve to fluctuations in ATP levels, may be an important defense mechanism that has been provided to the pump by nature.

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CHARACTERIZATION OF PIG KIDNEY Na,K-ATPase BY ANTIBODIES

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INTRODUCTION

In the standard procedure for preparation of purified Na,K-ATPase from outer medulla of pig kidney extraneous protein is extracted from the microsomal fraction of the homogenate by incubation with sodium dodecyl sulphate (SDS) in the presence of ATP (Jørgensen, 1974). Density gradient centrifugation in a zonal rotor next yields a fraction containing membrane-embedded Na,K-ATPase of high purity. SDS-polyacrylamide gel electrophoresis reveals only two protein bands, the catalytic peptide and the glycoprotein, α and β, respectively. Earlier estimates of binding capacities for ouabain and nucleotide were compatible with one molecule bound per 2 α chains, i.e. a dimeric structure of the enzyme. Transition between a protomeric and an oligomeric form of the enzyme is indicated from crystallization experiments (Jørgensen et al., 1982) and from nucleotide binding studies with native and solubilized enzyme (Jensen and Ottolenghi, 1983).

However, resolution of the membrane-bound Na,K-ATPase by means of the non-ionic detergent $C_{12}E_8$ into αβ-protomers with retained enzymatic activity (Brotherus et al., 1981) obviously means that the expected ligand binding capacity of the pure enzyme should be twice the observed value (Ottolenghi et al., 1986).

The discrepancy between the apparent electrophoretic purity and the deficiency in ligand binding capacity could be due to the presence of inactive peptides, e.g. excess of free α or β chains and denatured αβ-protomers, or unrelated proteins with the same electrophoretic mobility. In an attempt to solve the problem -

which is important from a mechanistic point of view of the pump - we tried to characterize enzyme preparations by means of antibodies raised against highly purified Na,K-ATPase from pig kidney outer medulla.

MATERIALS AND METHODS

Na,K-ATPase was prepared from pig kidney outer medulla as described by Jørgensen (1974), i.e. by selective extraction of unrelated proteins from the microsomal fraction with SDS in the presence of ATP followed by isopycnic zonal centrifugation. Fractions with highest specific activity were pooled and used as immunogen. The specific, ouabain-sensitive p-nitrophenyl phosphatase activity was 5.84 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ corresponding to an ouabain binding capacity of 4.14 $nmol \cdot (mg\ protein)^{-1}$ (Jensen et al., 1984).

In electro-immunoprecipitation experiments with selectively $C_{12}E_8$-extracted enzyme as antigen the starting material was an enzyme (E) with specific Na,K-ATPase activity of 35.5 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ corresponding to an ouabain binding capacity of 4.7 $nmol \cdot (mg\ protein)^{-1}$ (Jensen et al., 1984). The enzyme was treated with $C_{12}E_8$ as described by Jensen and Ottolenghi (1983) at a $C_{12}E_8$-to-protein ratio of 1.2 for 30 min at room temperature followed by centrifugation at 0°C at 100,000xg. The recovered supernatant containing purified enzyme with an expected ouabain binding capacity of about 6 $nmol \cdot (mg\ protein)^{-1}$ was used as antigen (E_o). For another immunodiffusion experiment Na,K-ATPase was chromatographied by ion-exchange on DEAE-Trisacryl M as described (Ottolenghi et al., 1986).

Na,K-ATPase was labelled with fluorescein isothiocyanate (FITC) at a concentration of 20 µM and a protein concentration of 1.4 $mg \cdot ml^{-1}$, pH 9.2, sedimented and washed at pH 8.0 as described by Hegyvary and Jørgensen (1981). Nucleotide binding to Na,K-ATPase, measurement of hydrolytic activity and SDS-polyacrylamid gel electrophoresis were carried out by established methods (Jensen and Ottolenghi, 1983).

A goat was immunized intracutaneously with Na,K-ATPase mixed with Freund's complete adjuvans for the first injection and incomplete adjuvans for successive immunizations following a schedule as described by Harboe and Ingild (1983). For each injection was given approximately 1 mg enzyme protein distributed at 3-5 injection points in the neck or shoulder. Bleedings were done

after 1 month and then every 2nd week for 4 months. Immunoglobulins were isolated from serum by salting out and ion-exchange chromatography (Harboe and Ingild, 1983).

Crossed immunoelectrophoresis and other electro-immunoprecipitations were carried out according to Axelsen (1983). The agarose gel contained 0.1 or 0.2 per cent of non-ionic detergent and as standard $C_{12}E_8$ (Bjerrum, 1983). Affinity electrophoresis was performed with both free and immobilized Concanavalin A inserted in the gel (for refs., see Axelsen, 1983). Immunoplates were stained for ATPase activity (Bjerrum, 1983) and phosphatase activity after careful washing and drying in cold air (for refs., see Axelsen, 1983). FITC-conjugated immunoprecipitates were inspected in UV-light before protein staining with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Any interference of the isolated immunoglobulin fraction with the hydrolytic activity of Na,K-ATPase was tested. It is seen from Fig. 1 that the antibodies inhibited overall ATP hydrolysis in the presence of $Na^+ + K^+$ but hardly the K^+-activated phosphatase activity. This might indicate that the antibodies 1)were directed against the substrate area of the enzyme, 2)gave rise to structural changes of the enzyme molecule or 3)simply gave rise to steric hindrance for the substrate. However, nucleotide binding studies revealed that neither the binding capacity nor the affinity of ^{14}C-labelled ADP was reduced by the immunoglobulins. Neither was phosphorylation by (^{32}P)ATP inhibited.

The individual bleedings carried out during the 5 months immunization period were tested by crossed immunoelectrophoresis (Fig. 2). Evolution of an immune response is obvious. The pattern shows two major peaks and occasionally feets of immunochemically related components probably due to denaturations (indicated by arrows in Fig. 3).

The double peak pattern was the aim for further analysis with purified pooled immunoglobulins of the antisera versus purified ATPase preparations. Two antibody preparations were made, one from the first bleeding and another from a pool of bleedings taken successively. Both these antibody preparations revealed very similar pattern (not shown), but traces of very small amounts of impurities were better detected by the second pool. Enzyme

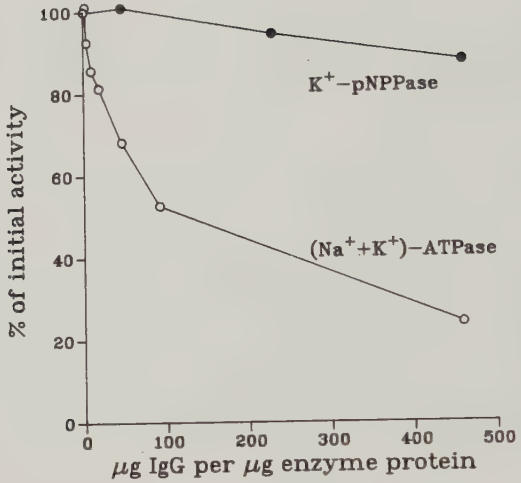

Fig. 1. Interference of immunoglobulins with hydrolytic activity of Na,K-ATPase. IgG and kidney enzyme (specific activity 29 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) were preincubated for 5 min at room temperature.

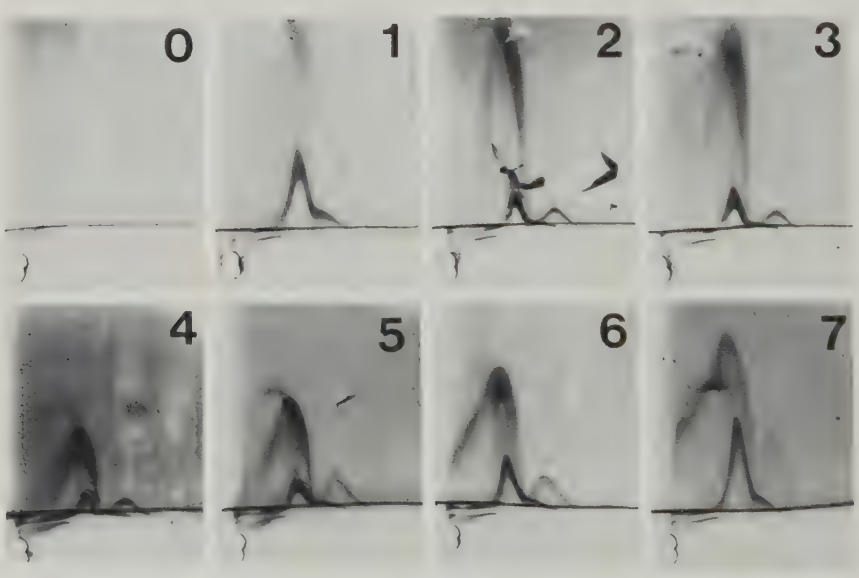

Fig. 2. Crossed immunoelectrophoretic analysis of the individual bleedings (1-7) from the goat immunized with pure pig kidney Na,K-ATPase.

extracted with $C_{12}E_8$ in order to further increase the purification also exhibited two prominent peaks. The double peak pattern can therefore not be due to contamination or denaturation, but must depict an immunochemical property within the enzyme preparation.

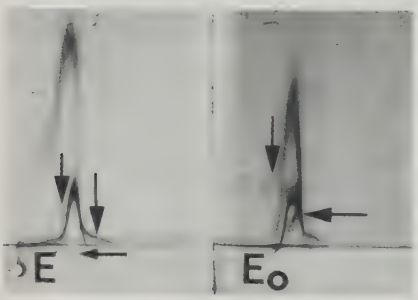

Fig. 3. Crossed immunoelectrophoresis with two highly purified ATPase samples (E and E_o) analysed with the purified fraction of pooled goat anti-pig Na,K-ATPase immunoglobulin. Note the denatured antigen (big arrows) and the small amounts of immunochemically unrelated impurities (small arrows). The prominent upper and lower peaks represent ATPase.

Any influence of the type and concentration of non-ionic detergent used for solubilization and inclusion in the gel was investigated in several experiments. The detergents Triton X-100, Nonidet P-40, Berol EMU, $C_{12}E_8$ and $C_{12}E_{10}$ were tested and similar immunoprecipitation patterns were obtained. The concentration of detergent during solubilization changed the ratio between the areas of the two major peaks (Fig. 4), but the concentration in the gel had only a slight effect on the pattern. However, in order to standardize the pattern we selected to use $C_{12}E_8$ since enzyme activity is preserved at low concentrations of this detergent.

In order to further characterize the pattern, we have attempted to perform histochemical staining for phosphatase and ATPase activity directly on the gel. Since the antibodies obviously inhibited enzyme activity and the solubilization also decreased activity it was only possible to reveal a slight phosphatase activity in the lower peak. This peak represents therefore $\alpha\beta$-unit(s). When FITC-labelled ATPase was used as antigen both peaks revealed fluorescence indicating that they contained the catalytic subunit. The fact that the lectin concanavalin A interacts with the β-subunit (Esmann, 1980) was used in affinity electrophoresis to identify the carbohydrate containing part of the ATPase molecule.

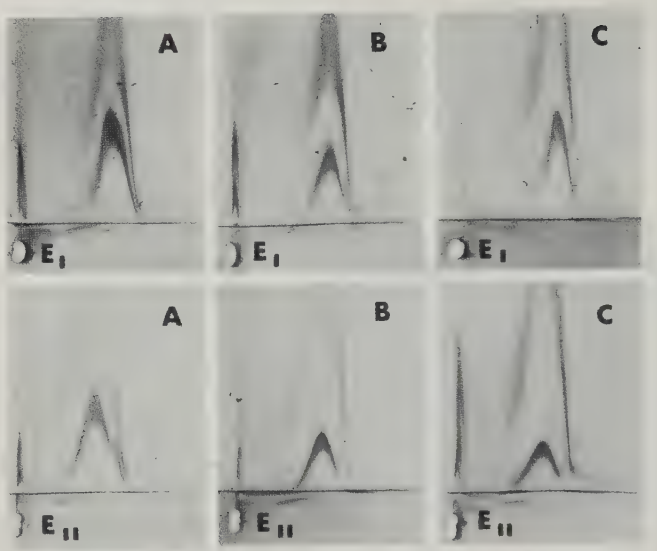

Fig. 4. Crossed immunoelectrophoretic analysis of two preparations of Na,K-ATPase (E_I og E_{II}) at different final concentration of $C_{12}E_{10}$ (A=1%, B=0.5%, C=0.1%) for sample solubilization. The gel contains 0.1% detergent.

Fig. 5 shows that only the lower peak expressed binding to Con. A. This unexpected observation has been reproduced in several experiments including affinity methods with the lectin inserted into an intermediate gel. Both free and sepharose-conjugated Con. A show affinities indicating that the β-chain has more than one binding site (Bøg-Hansen and Brogren, 1975). Similarly, the two peaks were separated after ion-exchange chromatography on DEAE-Trisacryl M (Ottolenghi et al., 1986).

Additional experiments have indicated, that the two components of ATPase indeed are immunochemical related but not identical. The lower peak (αβ) express a higher number of epitopes seen by the antibodies, than the upper component. This would be in agreement with the suggestion that the upper peak represents a free α-chain and the lower peak an αβ-protomer or dimer. Specific antibodies against the β-peptide would give the answer.

It is definitely not the antibody, that induces the diversity since the affinity binding of one peak to Con. A occurs in the first dimension electrophoresis where no antibody is present. The lectin might be able to split the αβ-unit, however, but then the relative

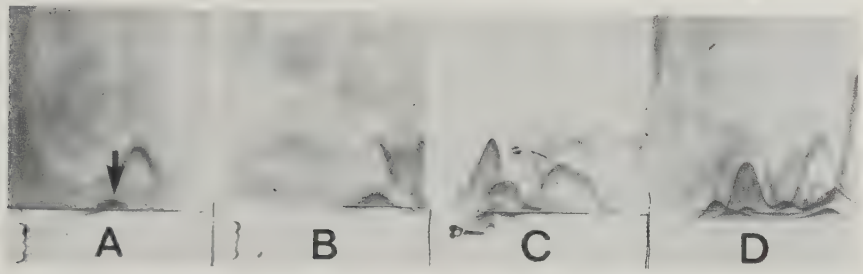

Fig. 5. Affinity immunoelectrophoresis with Con. A inserted in the 1st dimension zone electrophoresis. The pattern shows carbohydrate-containing β-chain present in the lower peak, but not in the upper peak. Plate A and C contained free Con. A inserted. Plate B and D were without lectin. In plate A and B the antigen was Na,K-ATPase, in plate C and D rat serum proteins versus their corresponding antibodies in the 2nd dimension gels.

peak size should be expected to change. A speculative possibility for this diversity could be an imbalance between the α- and the β-chain concentration in the solubilized preparation. Whether this is caused by the detergent or represents the situation also in the non-solubilized material is unknown. Such an imbalance in the membrane itself could have a regulatory function, the concentration of β-chains being the limiting factor for enzyme activity. In contrast to the related Ca-ATPase of SR, the Na,K-ATPase of plasma membranes needs the β-chain to express activity. The amount of β-chain inserted in the membranes in various tissues might therefore also regulate the pump capacity.

Since we cannot exclude that free β-chain might also be present in the solubilized samples, it remains to be proven with other methods, that there is an imbalance between the stoichiometric concentration of α- and β-chain in the native membrane. Quantitative HPLC-analyses might be useful for such comparisons.

In conclusion, immunochemical studies of highly purified Na,K-ATPase solubilized by non-ionic detergent have revealed the presence of multiple forms which might elucidate a regulatory role of the β-chain. The antibodies obtained seem highly specific for the pump proteins and proved a high degree of immunochemical purity of the enzyme preparations. Variations in actual enzymatic activity depict a change in homospecific activity probably due to variable denaturation during the preparation procedure.

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ASSOCIATION OF BIOCHEMICAL FUNCTIONS WITH SPECIFIC SUBUNIT ARRANGEMENTS IN PURIFIED Na,K-ATPase

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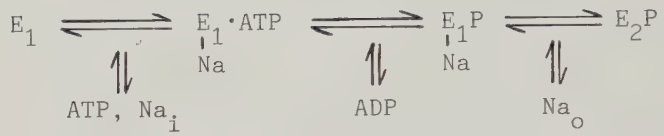
There is conflicting evidence regarding the possibility that the normal operation of Na,K-ATPase requires multiple and interacting subunits (Askari & Huang, 1985; Glynn & Karlish, 1975). Of the methods available to estimate molecular size, radiation inactivation has been widely used as the one which returns the size of the enzymatically active particle (Kepner & Macey, 1968; Harmon et al., 1985). The basic principle of the method is that the larger the target size the more likely it is to be annihilated by a constant barrage of very small, highly penetrating particles. Using this technique, the target size for the Na,K-ATPase activity generally appears greater than the sum of α and β-chains (Kepner & Macey, 1968; Ottolenghi et al., 1983; Schrijen et al., 1983). On the other hand, using conventional hydrodynamic methods, the molecular size of one solubilized preparation with Na,K-ATPase activity has been found to be close to the sum of α and β polypeptides (Brotherus et al., 1983). However, despite the fact that the α-chain contains the ATP-binding site and the phosphorylation site (Jørgensen, 1982), a partial reaction supported by the isolated large polypeptide has never been reported. The present experiments strongly suggest that the α-chain can on its own catalyse all the initial, Na-dependent reactions of Na,K-ATPase.

ATP-ADP EXCHANGE: THE FIRST HALF-REACTION

ATP-ADP exchange appears as a consequence of the reversible phosphorylation of the pump by ATP,



In this case, the exchange has been measured in a high-Na medium and in the absence of K ions. In these conditions, its rate not only depends on Na^+ binding at the "intracellular" Na-loading sites, but is increased by Na^+ acting at low-affinity "extracellular" sites. As the scheme illustrates, this requires a reversal of the conformational change in the phosphoenzyme (Cavieres, 1980; Kaplan & Hollis, 1980).

PROCEDURE

The enzyme was purified from pig kidneys (Jørgensen, 1974) and had a Na,K-ATPase activity of 14-16 $\mu moles \cdot min^{-1} \cdot mg\ protein^{-1}$. ATP-ADP exchange was measured backwards as the incorporation of label from [^{3}H]-ADP into ATP; it was corrected for ATP hydrolysis by measuring the Na-ATPase activity with [γ^{32}P]-ATP simultaneously and in the same tubes (Cavieres, 1983, 1987). The reaction medium contained 150 mM NaCl, 25 mM histidine (pH 7.4), 0.1 mM EGTA, 0.33 mM $MgCl_2$, 1.5 mM ADP (plus [^{3}H]-ADP) and 1.5 mM ATP (plus [γ^{32}P]-ATP) and membranes at 55 µg protein/ml. The calculated Mg^{2+} concentration was around 20 µM. To measure the target size, enzyme samples in 250 mM sucrose, 25 mM histidine (pH 7.4) and 1 mM EDTA were freeze-dried and irradiated with 16 MeV electrons (Addenbrooke's Hospital, Cambridge) under vacuum and at room temperature. ATP-ADP exchange was measured as a time-course after reconstitution and dilution of the samples (Cavieres, 1987).

THE TARGET SIZE FOR ATP-ADP EXCHANGE

Figure 1 shows the result of one of two experiments. The ordinate shows the percent of the sample ^{3}H appearing in the ATP fraction (top) or the percent of the sample ^{32}P in the AMP/P_i fraction (bottom). Each boiled sample yields one % [^{3}H]-ATP and one % $^{32}P_i$. The Na-ATPase rates were calculated by linear regression of the data sets in the lower panel. The ATP-ADP exchange rate-constant was obtained from the slope of semilogarithmic plots of (1 - [^{3}H]-ATP/[^{3}H]-ATP_∞)

vs. time (Cavieres, 1987).

The inactivation plot for these data is shown in Fig. 2. The control activities were (μmoles·min^{-1}·mg protein^{-1}, mean ± S.E.M.) 0.764 ± 0.009 for ATP-ADP exchange and 0.645 ± 0.012 for the Na-ATPase activity. Considering the two experiments, the average target size was 118 kDa for ATP-ADP exchange and 201 kDa for the Na-ATPase activity, respectively. Evidently, the particle size for ATP-ADP exchange is quite close to the molecular weight of the α-chain, as calculated from the cDNA sequence (Shull et al., 1985). Since these

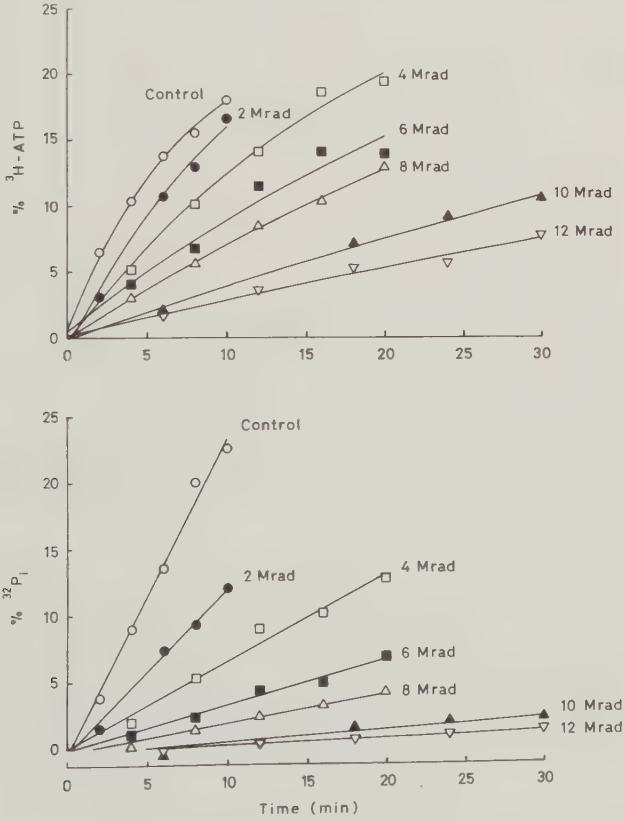


FIGURE 1. Simultaneous time-courses of ATP-ADP exchange (top) and Na-ATPase activity (bottom) of control and irradiated samples of purified Na pump. Curves drawn as described in an earlier article (Cavieres, 1987).

experiments were conducted at a high Na concentration, the exchange rate should at least partially depend on the rate of reversal of the conformational transition of the phosphoenzyme. Being the exchange inactivation-curve a single exponential, it follows that isolated α-chains should be able to perform reversibly the string of Na-dependent partial reactions from E_1 to E_2P. On the other hand, the Na-ATPase activity of the Na pump is thought to arise from the spontaneous breakdown of the phosphoenzyme in the absence of K ions (Post et al., 1972). The target size is greater than the sum of α and β chains (cf Ottolenghi et al., 1983), but less than that for α_2, more like $\alpha\beta_2$.

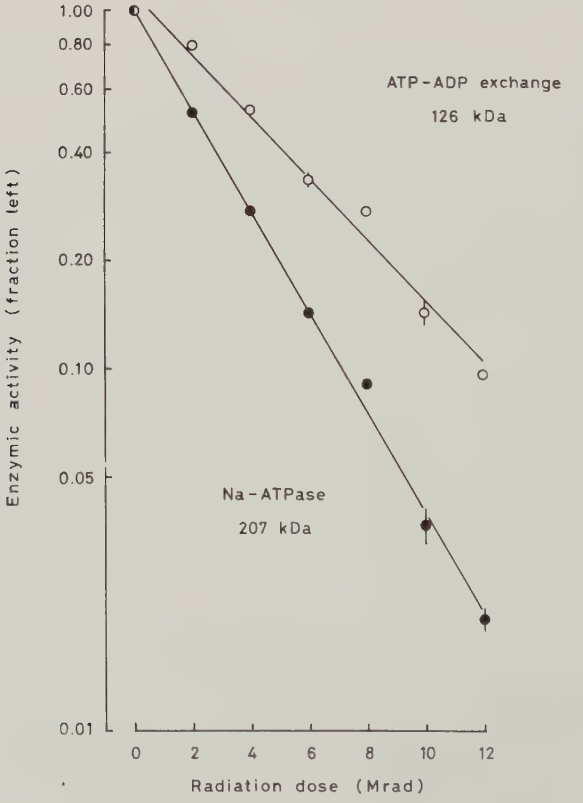


FIGURE 2. Radiation inactivation of ATP-ADP exchange and Na-ATPase activities. The slopes (base e) of the regression lines are ($Mrad^{-1}$) -0.1982 ± 0.0039 (ATP-ADP exchange) and -0.3231 ± 0.0032 (Na-ATPase). Vertical bars represent ± 1 S.E.M.

The existence of such asymmetrical array of target sizes does not necessarily imply that isolated α-chains roam freely in the plane of the membrane. All we can say is that, having the irradiation deprived an α subunit from all eventual partners (including other α subunits), the survivor can perform through all the Na-dependent steps of the overall reaction and no further. In other words, that the α-chain is the minimal unit for ATP-ADP exchange, but is insufficient for hydrolysis.

Recent experiments from this laboratory (submitted for publication) show that whereas at 1 mM ATP a target size of 241 kDa is obtained for the Na,K-ATPase activity, it is only 173 kDa when the same samples are assayed at 10 μM ATP. Thus, the αβ protomer seems enough to catalyse the slow, K^+-inhibited hydrolysis which obtains when only the high-affinity ATP sites are involved. But for the 20-fold stimulation arising from the occupation of low-affinity ATP sites, a larger particle seems required and α_2, or perhaps $\alpha_2\beta$, can be suggested.

CONCLUSIONS

Starting from E_1, increasingly larger particles seem necessary to support the various partial reactions round the Na pump cycle. Thus, whereas α-chains are able to catalyse reversibly the reactions steps leading to E_2P, a particle the size of α_2 or larger seems necessary to obtain Na,K-ATPase activity at 1 mM ATP. Particles of intermediate size can support K-independent hydrolysis or Na,K-ATPase activity at 10 μM ATP.

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TARGET-RESIDUES OF THE ACTIVE SITE AFFINITY MODIFICATION ARE DIFFERENT IN E_1 AND E_2 FORMS

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INTRODUCTION

Advances in the study of Na^+,K^+-ATPase functioning mechanism at the molecular level initiated investigations of the structural elements of the enzyme's functionally important regions, especially the active center. Enzymatic and ion-translocating functions and their close integration are accompanied by conformational transition between two states E_1 and E_2, for all transport ATPases of the E_1-E_2 type, including Na^+,K^+-ATPase. Conformational transition gives rise to a pronounced change in substrate affinity (Post et al., 1972). It means that the E_1-E_2 transition includes changes in the structural organization of the active site as an obligatory step in the enzyme reaction. Enzyme affinity modification with substrate analogs, containing active groups capable of covalent bonding to the sites within the active center is a promising approach to this problem.

ATP-γ-[4-(N-2-chloroethyl-N-methylamino)] benzylamid (C1RATP) was shown to be a Na^+,K^+-ATPase functional substrate analog. C1RATP covalent binding to the functionally important amino acid residue of the α-subunit resulted in complete inhibition of the ATP-hydrolysing activity (Dzhandzhugazyan et al., 1984). This reagent was used to study an oligomeric organization of Na^+,K^+-ATPase (Dzhandzhugazyan and Modyanov, 1985). Specific incorporation leading to complete enzyme inactivation in the Na-form required one C1RATP molecule per $(\alpha\beta)_4$-tetramer, but in the K-form only one C1RATP molecule per αβ-protomer. The present

work describes the isolation of α-subunit fragments, including C1RATP modified regions in E_1 and E_2-forms of Na^+,K^+-ATPase, and their structural determination. Modification sites, different for E_1- and E_2-enzyme states, were established. A model of the spatial arrangement of Na^+,K^+-ATPase active site was proposed.

RESULTS AND DISCUSSION

Fig. 1 outlines the results of Na^+,K^+-ATPase inhibition with C1RATP in the presence of 150 mM NaCl or KCl and prevention of its inactivation with ATP.

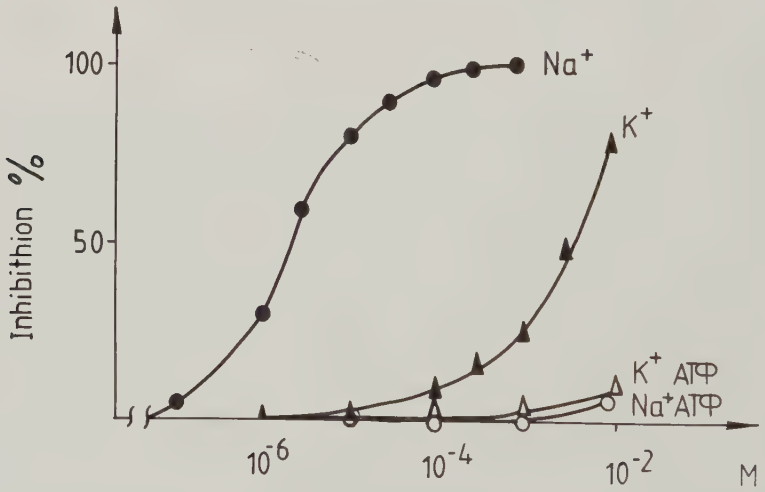


Figure 1. Dependence of the inactivation of Na^+,K^+-ATPase on C1RATP concentration in the presence of 150 mM NaCl (●), 150 mM KCl (▲), 150 mM NaCl and 10 mM ATP (o), 150 mM KCl and 10 mM ATP (△). The reaction mixture was incubated for 30 min. at 37°C.

As a consequence of drastic decrease in the enzymes affinity for C1RATP in the E_2-state, inhibition in the presence of K^+ occurs at the higher reagent concentration as compared to Na^+. Nevertheless at the same extent of inhibition the K-form of the enzyme is characterized by higher stoichiometry of specific, i.e. ATP-protected, incorporation of the reagent (1 molecule per protomer at full inactiva-

tion) (Dzhandzhugazyan et al., 1984). Hence, the K-form is preferable for isolating the modified fragment, but the high reagent concentration (5mM C1RATP) required for pronounced (~60%) inactivation resulted in high nonspecific modification. We successfully avoided that difficulty by differential modification, where modification of the enzyme started only with 4-(N-2-chloroethyl-N-methylamino)benzylamine (C1R) in the presence of ATP. The second stage involved [^{14}C] C1RATP, containing the [^{14}C]label in the benzyl group. So, the radioactive label was a marker of the active site region (Dzhandzhugazyan et al., 1986). In case of the Na-form, direct and differential modifications lead to the same results. Both the products in Na- and K-forms were stable in the membranebound enzyme and became temperature- and alkaline labile after denaturation with 1% SDS. This allowed us to presume the ester to be the modification product. That was proved later when we compared the modification of isolated peptides and a model compound - ester of C1R and acetic acid (Dzhandzhugazyan et al., 1986). Hydrolysis of membrane-bound modified Na^+,K^+-ATPase with pepsin at pH 2, 4°C, for 96 hrs. was chosen to keep the product stable.

Modified peptides were isolated according to the same procedure: first reverse phase HPLC was used to separate pepsin hydrolyzate (Fig. 2), then microbore reverse phase HPLC for rechromatography (Fig. 3). Radioactive label distribution, light absorption at 260 nm and spectral characteristics for C1R (ATP splits off C1RATP at proteolysis since the phosphoamide bond is labile in acidic solution) proved the modification of the isolated peptide. Comparison of chromatografic characteristics of both the peptides showed the peptides to be different - the Na-peptide had less retention time on the reverse phase column and less ratio of absorption at 260 nm and 210 nm (Fig. 3).

Amino acid sequences of the Na- and K-peptides, Ala-Val-Thr-Gly-Asp-Gly-Val-Asn and Ala-Val-Thr-Gly-Asp-Gly-Val-Asn-Asp-Ser-Pro-Ala-Leu, respectively, are identical in their N-terminal sequence, but the K-peptide has extra amino acids in the C-terminus and contains two Asp residues, Asp^{710} and Asp^{714}, which are potential modification sites.

The K-peptide amounts measured relative to the amino-terminal residue and its radioactivity and/or absorption at 260 nm were compared and showed only one modified residue in the peptide.

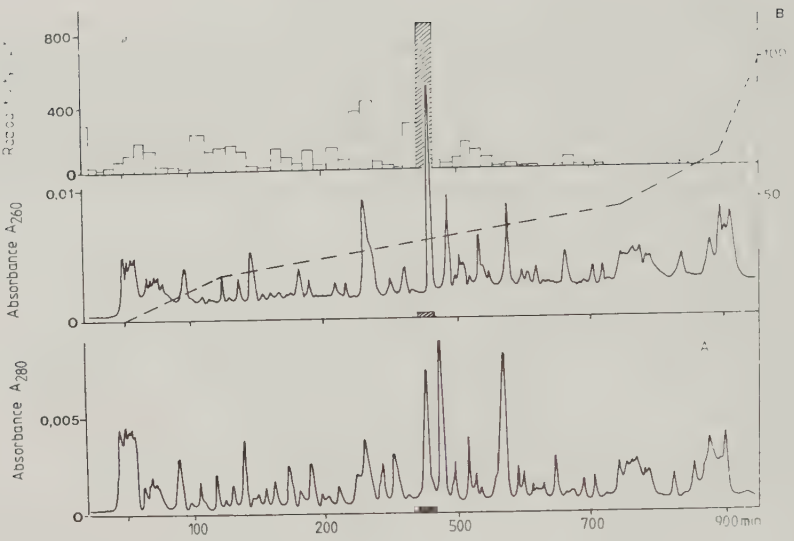


Figure 2. Separation of peptides from the pepsin hydrolyzate and distribution of radioactivity along the Bakerbond Wide Pore C_{18} column (0.46x25 cm). Eluting buffers: A- 0.1% TFA, triethylamine was added up to pH 2.5, B- 50% CH_3CN in buffer A. Flow rate 0.2 ml/min. Upwards: A- absorbance at 280 nm, B- at 260 nm, broken line - change in percent content of buffer B. The height of the columns (C) corresponds to radioactivity of the fraction. The fraction subjected to further chromatography is hatched.

Two-fold increase of hydrolysis time did not result in degradation of the K-peptide to the Na-peptide. That pointed to modification of Asp^{714}, which prevented hydrolysis of bond Asn(713)-Asp(714). Indeed, computer prediction (Antonov et al., 1977) of bonds, preferentially split by pepsin, showed that only one bond Asn(713)-Asp(714) tended to be hydrolyzed. When Asp(714) was substituted with Lys or Arg (which contained an amino group as a modification product), the probability of the bond cleavage falls to 0. Thus, Asp(714) of the α-subunit might be the target of modification of Na^+,K^+-ATPase in the E_2-form. Region Ala(706)-Leu(718) is a part of the active center of Na^+,K^+-ATPase.

Its conformational transisitons is the basis for Asp(710) or Asp(714) modification in the E_1- and E_2-form. Comparison of the amino acid sequence of the peptide and the corresponding regions of the α-subunit of all the known ATPases of the E_1-E_2-type shows the high sequence homology of the fragments; both functionally important Asp residues are invariant in all these structures. The homology region, including the isolated peptides, is rather extended and embraces also the fragment of the polypeptide chain modified with 5'-(p-fluorosulfonyl)benzoyladenosine (Nagano et al., 1986). Here Lys, being a modification target, is invariant in all the mentioned ATPases except for Kdp ATPase of E.coli (Serrano et al., 1986). Recently, two other residues involved in formation of the active center were determined - Asp (369), P_i-acceptor in the ATPase reaction (Walderhaug et al., 1985), and Lys(501), selectively modified by FITC (Farley et al., 1984).

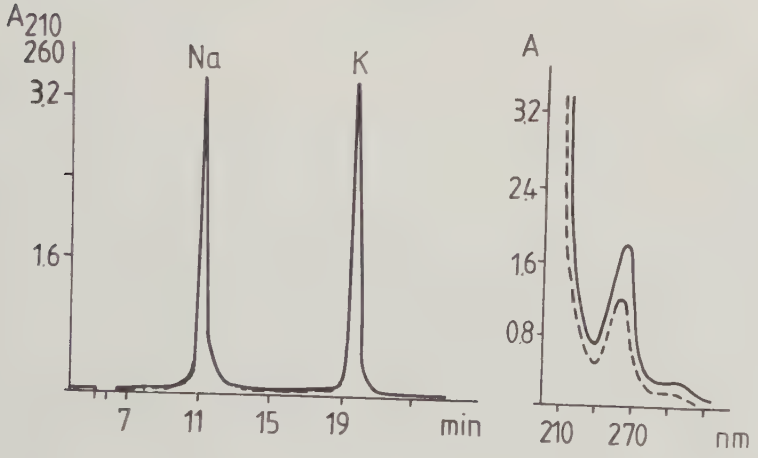
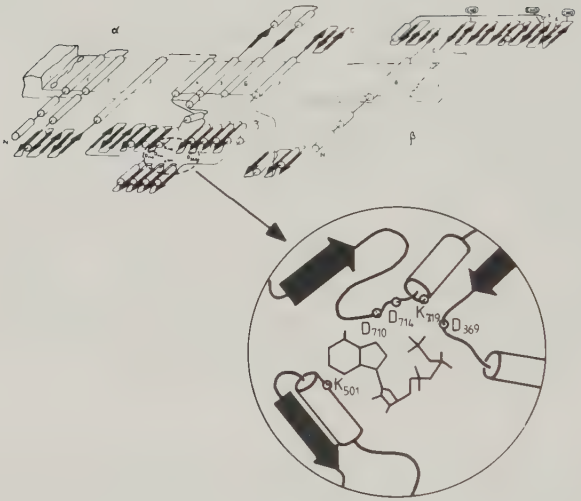


Figure 3. Comparison of the retention times and spectra of the K-peptide (dotted line) and Na-peptide. (Column 0.2x6.4 cm, Nucleosil $5C_{18}$, stepwise gradient CH_3CN in buffer A: 100 µl buffer A, 700 µl 10% CH_3CN, 700 µl 20% CH_3CN; flow rate 50 µl/min.).



Na^+,K^+-ATPase	^{706}Ala-Val-Thr-Gly-Asp-Gly-Val-Asn- Asp-Ser-Pro-Ala-Leu^{718}
Ca^{++}-ATPase	^{698}Ala-Met-Thr-Gly-Asp-Gly-Val-Asn- Asp-Ala-Pro-Ala-Leu^{710}
H^+-ATPase	^{630}Ala-Met-Thr-Gly-Asp-Gly-Val-Asn- Asp-Ala-Pro-Ser-Leu^{642}
yeast plasma membrane	
KDP-ATPase	^{512}Ala-Met-Thr-Gly-Asp-Gly-Thr-Asn- Asp-Ala-Pro-Ala-Leu^{524}

Figure 4. The model of spatial organization of the Na^+,K^+-ATPase. The active center, with functionally important amino acids residues, is encircled by a dotted line. The arrangement of the regions neighbouring ATP and a comparison of amino acid sequences of the modified peptides and corresponding regions of same E_1–E_2 ATPase are also shown.

Fig. 4 presents the model for spatial organisation of the Na^+,K^+-ATPase α-subunit based on experimental and computed prediction data on the α-subunits secondary structure (Arzamazova et al., this volume). The active center, with functionally important amino acid residues mentioned above, is dotted. Both Asp(710), Asp(714), shown in the work to be sites of modification by C1RATP in forms E_1 and E_2, and Asp (369) are located in loops of the polypeptide chain. These loops are apart from any structural domains and, apparently, conformationally flexible. At the level of structural perturbations in the active center, the E_1-E_2 transition manifests itself in small changes of the geometry of a relative arrangement of the substrate and the polypeptide chain loop, containing both functionally important residues Asp(710) and Asp(714).

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A REVISED BOUNDARY LIPID COUNT FOR Na,K-ATPase FROM SQUALUS ACANTHIAS

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INTRODUCTION

Lipid-protein interactions in Na,K-ATPase rich membranes from Squalus acanthias have been studied using spin-labelled lipids in conjunction with electron spin resonance (ESR) spectroscopy (Esmann et al., 1985; Esmann and Marsh, 1985). These studies, employing a number of spin-labelled phospholipids, showed the presence of a motionally restricted component in the ESR spectra in addition to a component which corresponds closely to the ESR spectra obtained from dispersions of the extracted lipid (Esmann et al., 1985). The pH dependence and salt dependence of the lipid-protein interaction of negatively charged lipids (phosphatidic acid, phosphatidylserine and stearic acid) has also been studied using the ESR technique, and it was observed that direct electrostatic effects alone cannot account for the selectivity of negatively charged lipids for the protein (Esmann and Marsh, 1985).

The purpose of the present paper is to show that a motionally restricted component also is observed when a pyrrolidine-based spin-labelled fatty acid is used instead of the usual doxyl nitroxides. In the pyrrolidine-based fatty acid the nitroxide moiety forms part of the hydrocarbon chain, figures 1 and 2. This structure perturbs chain packing less than the conventional doxyl labels, figures 1 and 2, and in addition, the label axes are differently

oriented, which gives rise to a quite different sensitivity of the ESR spectra to molecular rotational motion. The latter constitutes a critical test for the existence of a protein associated lipid population with reduced mobility and of the spectral subtraction methods used for quantitating the motionally restricted components.

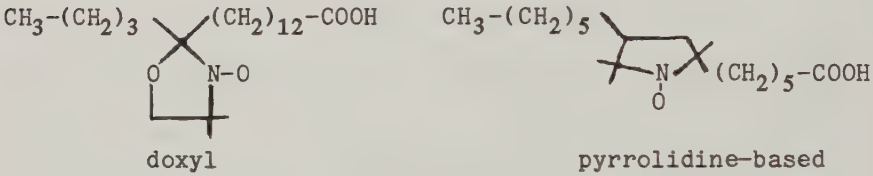

Figure 1. Structures of the doxyl spin-labelled stearic acid (14-(4,4-dimethyloxazolidine-N-oxyl)stearic acid) and the pyrrolidine-based fatty acid (2,5,5-trimethyl-4-hexyl-2-(5-carboxypentyl)-pyrrolidine-N-oxyl).

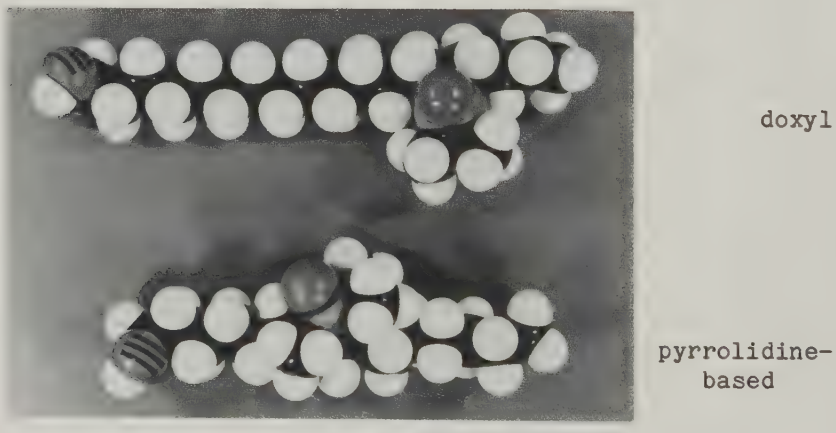

Figure 2. Space filling models (PCK) of the two spin-labels, the doxyl labelled stearic acid and the pyrrolidine-based fatty acid spin-label.

METHODS

Na,K-ATPase-rich membranes were prepared according to

the method of Skou and Esmann (1979), and the preparations used contained typically 50-70% Na,K-ATPase, estimated from the protein content (i.e. the amount of α- and β-subunits) and from the specific Na,K-ATPase activity, which ranged from 1200 to 1700 μmoles ATP hydrolysed/mg protein per hour.

The doxyl stearic acid spin-label with the nitroxide in the C-14 position (figures 1 and 2, left hand side) was synthesised according to the method of Hubbell and McConnell (1971). The pyrrolidine-based spin-label (figures 1 and 2, right hand side) was synthesised as described by Hideg and Lex (1984). The spin-labels were introduced into the membrane suspension in 100 mM NaCl, 1 mM CDTA and 30 mM histidine (pH 7.4) at a level of 1 mol % relative to membrane lipid from a small volume of concentrated solution in ethanol. After incubation overnight excess label was removed by centrifugation, the pellet resuspended either in the same buffer (for stearic acid) or in 10 mM Tris (pH 9.2) (for the pyrrolidine based spin-label) and the centrifugation was repeated. The resultant pellet was taken up into a glass capillary and used for ESR spectroscopy. Lipid dispersions were prepared as previously described (Esmann et al., 1985). ESR spectra were recorded on a Varian E-12 9-GHz spectrometer as previously described (Esmann et al., 1985).

RESULTS

The method for analyzing the two-component spectra by digital subtraction is illustrated for the doxyl stearic acid spin-label in figure 3. A motionally restricted spin-label component (indicated by the arrows in figure 3a) is clearly seen in the outer wings of the membrane spectrum, and is not present in the spectrum of the lipids alone (figure 3b). Subtraction of the lipid spectrum from the membrane spectrum yields the spectrum of the motionally restricted component (figure 3d), and double integration gives the fraction of the total intensity (34%) which is present in this component. Subtraction of the motionally restricted component from the membrane spectra, using a matching spectrum from dimyristoylphosphatidylcholine vesicles in the gel phase, yields the spectrum of the fluid component (figure 3c) which is closely similar to that of the extracted lipid. Double integration yields a relative intensity of 66 % for this fluid component, in agreement with the quantitation from the motionally restricted compo-

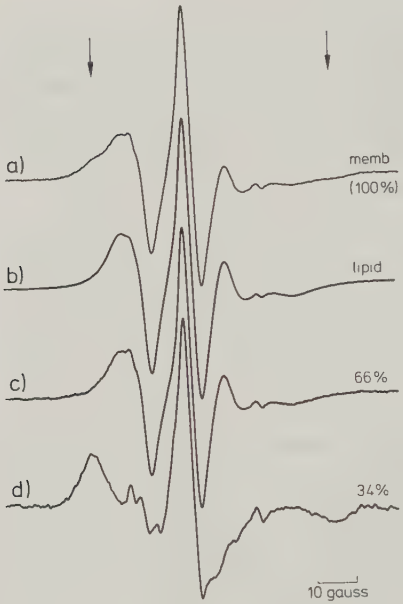

Figure 3

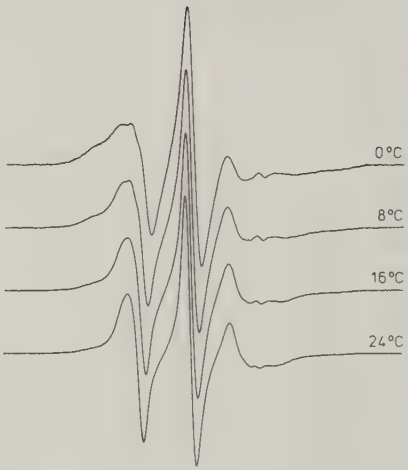

Figure 4

Figure 3. Spectral subtraction of the composite ESR spectrum of the C-14 doxyl stearic acid spin-label in Na,K-ATPase membranes at 0 °C. a) spectrum of the stearic acid spin-label in native membranes; b) spin-label in extracted lipids; c) fluid difference spectrum obtained by subtracting a motionally restricted spectrum (dimyristoylphosphatidylcholine vesicles in the gel phase) from a); d) motionally restricted spectrum obtained by subtracting b) from a). Total scan width is 100 G and the arrows indicate the position of the motionally restricted component. Buffer used : 100 mM NaCl, 20 mM histidine and 1 mM CDTA (pH 7.4).

Figure 4. Temperature dependence of the ESR spectra of the stearic acid spin-label in Na,K-ATPase membranes. Buffer used : 100 mM NaCl, 20 mM histidine and 1 mM CDTA (pH 7.4). Total scan width 100 G.

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nent.

The spectral line shape of the doxyl stearic acid spin-

label in Na,K-ATPase membranes changes considerably with temperature as indicated in figure 4. The largest changes occur in the fluid component, which gets considerably sharper as the temperature increases. For this reason the fluid component begins to dominate over the motionally restricted component at higher temperatures, but the latter component is still observable in the spectral wings. There is actually only a small decrease in the fraction of motionally restricted component at 24 ^{o}C relative to 0 ^{o}C (Esmann et al., 1985).

Figure 5 shows a similar analysis of the pyrrolidine-based spin-label in Na,K-ATPase membranes, here at pH 9.2. The method of subtraction is analogous to the method used for stearic acid (figure 3), and the motionally restricted component constitutes 49% of the total intensity. The higher factor obtained here relative to that of the stearic acid label is due to the higher pH used. The deprotonated form of the acid, which is predominant at high pH, has a higher selectivity for the protein than the protonated form (Esmann and Marsh, 1985).

The temperature dependence of the pyrrolidine-based spin-label is shown in figure 6. The spectral lineshape changes also here with temperature, but the motionally restricted component is clearly present in the spectral wings also at high temperatures.

Previous spin-label studies have revealed a selectivity of the lipid-protein interaction in the order cardiolipin > phosphatidylserine ~ stearic acid ~ phosphatidic acid > phosphatidylglycerol ~ phosphatidylethanolamine ~ phosphatidylcholine. The value for the fraction of motionally restricted component for the non-selective lipids is about 20%-22% of the total intensity (Marsh et al., 1982; Esmann et al., 1985). The lipid/protein weight ratio is about 0.87 in this preparation (Esmann et al., 1985), and the number of motionally restricted lipid molecules (M_r 700) is thus about 0.20-0.22 mmol/g protein. Using a molecular weight of 147 000 D for the $\alpha\beta$-protomer a value of about 30-33 mol lipid per mol protomer is obtained.

The 3-dimensional structure at low resolution has been determined for 2-dimensional crystals of kidney Na,K-ATPase (Hebert et al., 1985). Figure 7 shows the contour map of the protein in the intramembraneous section of two protomers. At

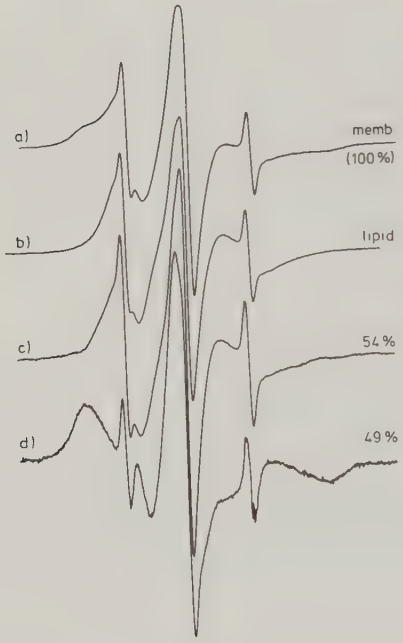

Figure 5

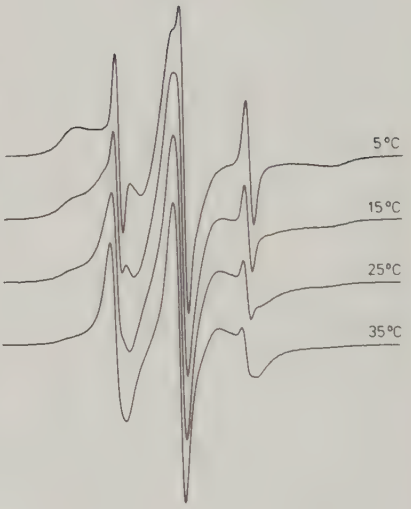

Figure 6

Figure 5. Spectral subtraction of the composite ESR spectrum of the pyrrolidine-based fatty acid spin-label in Na,K-ATPase membranes at 0 ^{o}C. a) spectrum of the fatty acid spin-label in native membranes; b) spin-label in extracted lipids; c) fluid difference spectrum obtained by subtracting a motionally restricted spectrum (dimyristoylphosphatidylcholine vesicles in the gel phase) from a); d) motionally restricted spectrum obtained by subtracting b) from a). Total scan width 100 G. Buffer used : 10 mM Tris (pH 9.2).

Figure 6. Temperature dependence of the ESR spectra of the pyrrolidine-based fatty acid spin-label in Na,K-ATPase membranes. Buffer used : 10 mM Tris (pH 9.2). Total scan width 100 G.

the protein/lipid interface 36 hydrocarbon chains can be fitted around each protomer. With two hydrocarbon chains per phospholipid molecule (and lipid annuli on both sides of the membrane) we obtain a lipid/protein molar ratio of 36, which

is in good agreement with the value of 30-33 mol/mol obtained from the quantitation of the motionally restricted component (see above).

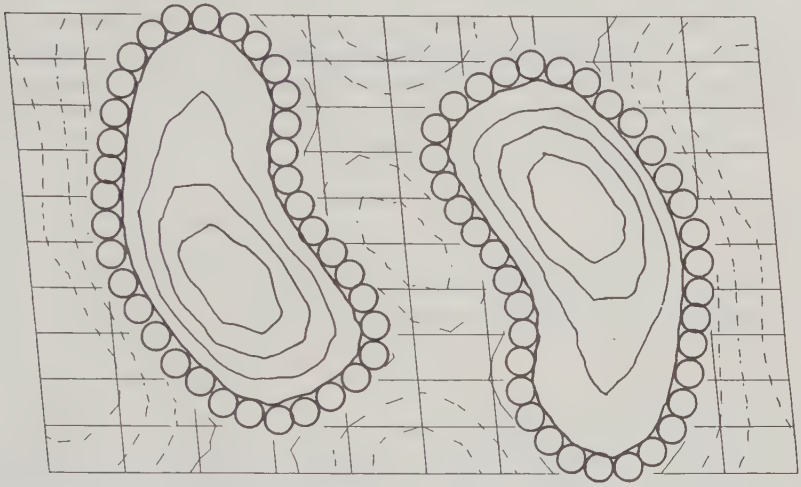

Figure 7. Boundary lipids on the Na,K-ATPase. Lipid chains (circles of diameter 4.8 Å) are fitted around the intramembranous section of the αβ-protomer using the data of Hebert et al., (1985). There is room for 36 chains per protomer. The perimeter chosen for the protein corresponds to the 10 Å-level in the 3-dimensional model for crystallized Na,K-ATPase (see figure 5 in Hebert et al., 1985).

CONCLUSION

A motionally restricted protein-associated lipid component has been observed in the ESR spectrum of Na,K-ATPase membranes using two different spin-labelled fatty acids. The spin-label group modifies the structure of the parent fatty acid in quite different ways, and gives rise to quite different ESR spectra. Nonetheless, the fraction of motionally restricted spin-label component at high pH is rather similar for the two derivatives: 49% for the pyrrolidine-based fatty acid and 42% for the doxyl fatty acid (Esmann and Marsh, 1985). Thus any possible perturbing effects of the spin-label are not responsible for the observation of the motionally restricted component, nor do they appreciably affect the quantitation of this component.

The most recently avaible structural information (Hebert et al., 1985) has been used to reestimate the number of lipid chains which can be accomodated around the intramembraneous section of the Na,K-ATPase. It is concluded that approximately 36 lipid molecules can be accomodated around each αβ-protomer, which correlates quite well with previous determinations of the fraction of motionally restricted spin-labelled phosphatidylcholine which is associated with the membraneous enzyme.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Elisabeth Skriver for providing the contour map used in figure 7.

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DISTANCES BETWEEN 5-IODOACETAMIDOFLUORESCEIN AND THE ATP AND OUABAIN SITES OF (Na,K)-ATPase DETERMINED BY FLUORESCENCE ENERGY TRANSFER

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INTRODUCTION

The fluorescent probe 5-iodoacetamidofluorescein (IAF) labels the α-subunit of (Na,K)-ATPase covalently without perturbation of its enzymatic activity (Kapakos and Steinberg, 1982). The IAF-labelled enzyme shows fluorescence changes associated with various conformational transitions (Kapakos and Steinberg, 1986a,b), including those that accompany the occlusion and release of K or Rb ions (Glynn et al., 1987).

In the present work we determined the stoichiometry and specificity of labelling with IAF, and we deduced the location of IAF with respect to the ouabain and ATP sites from measurements of fluorescence energy transfer between IAF and fluorescent analogues of ATP and ouabain bound specifically to their respective sites on native (Na,K)-ATPase purified from dog kidney. The ouabain site was labelled with anthroylouabain (AO), which binds to this site with affinity and specificity similar to ouabain (Fortes, 1977). The ATP site was labelled with trinitrophenyl-ATP (TNP-ATP), which binds to the ATP site with high specificity and with higher affinity than ATP (Moczydlowski and Fortes, 1981a,b).

METHODS

(Na,K)-ATPase was purified from dog kidney and labelled with IAF exactly as described by Kapakos and Steinberg (1982). The IAF content was determined by absorbance (Kapakos and Steinberg, 1982), or fluorimetrically, using as a standard the fluorescence of samples of known IAF content,

determined by absorbance. The protein was determined by the method of Lowry and corrected by the differences between this method and quantitative aminoacid analysis as described by Lee and Fortes (1986).

Determinations of active site concentrations were done as described by Fortes (1986). Steady-state and time-resolved fluorescence measurements, and calculation of distances from energy transfer data were done as described by Lee and Fortes (1986).

RESULTS AND DISCUSSION

Evidence that IAF labels predominantly a single site

The IAF-labelling stoichiometries ranged from 6.8 to 8.8 nmoles/mg protein, which correspond to 1-1.3 mol IAF per mol αβ (Mr=147 000, Lee and Fortes, 1986). The upper values included some unreacted IAF, as evidenced by a fluorescent band at the gel front which could be removed by extensive washing. Labelling with IAF was prevented by preincubation of the enzyme with 15 mM iodoacetate at 37°C for 30 min. These conditions have been shown by Castro and Farley (1979) to cause carboxymethylation of a single residue of the α-subunit, located in a fragment of about 18 kDa between the sites of trypsin and chymotrypsin cleavage. The inhibition of IAF-labelling by iodoacetate, and the IAF-labelling stoichiometries of about 1 mole IAF per mole of αβ suggest that most or all the IAF-labelling was at the iodoacetate-reactive site, probably a cys residue.

Distance between AO and IAF

Fluorescence titrations of unlabelled and IAF-labelled (Na,K)-ATPase showed that the affinity for AO was unaffected by IAF, but the AO quantum yield was decreased in the IAF-labelled preparations (Fig.1). Emission spectra showed that the decreased AO quantum yield in the IAF-labelled preparations was accompanied by sensitized emission of IAF. Thus, the quenching of AO fluorescence observed in the IAF-labelled enzyme was due to energy transfer and not to a perturbation of the AO site caused by the labelling.

The efficiency of energy transfer from AO to IAF, determined from the ratio of the initial slopes of

titrations as in Fig. 1, was 15 to 25% in different preparations. The distance for 50% energy transfer, calculated from the spectroscopic parameters of AO and IAF and the assumption of random orientations of these probes, was 42 Å. The distance between the IAF-labelled site and the ouabain site, estimated from these values, was 50 to 56 Å. This distance indicates that the IAF-reactive site is on the cytoplasmic portion of the enzyme, and is consistent with previous energy transfer studies which suggested that all the -SH groups labelled with fluorescein mercuric acetate were more than 45 Å from the AO site, at an average distance of 72 Å from the AO site (Jesaitis and Fortes, 1980).

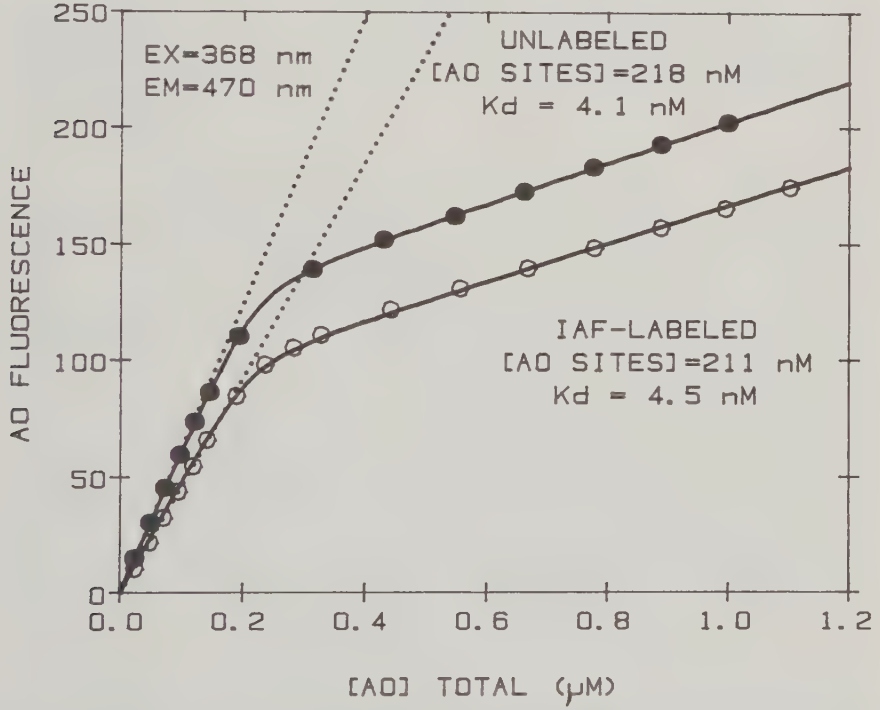

Figure 1. AO titrations of IAF-labelled and unlabelled (Na,K)-ATPase. Conditions: 50 mM Pipes-tris, 0.1 mM CDTA, 6 mM $MgCl_2$, 6 mM Pi-tris, pH 7.0 and (●) 91 μg/ml unlabelled or (○) 75 μg/ml IAF-labelled (Na,K)-ATPase. The initial slopes (dotted lines) are proportional to the quantum yields of bound AO. The solid lines are the best fits for AO binding to a homogeneous site (Fortes, 1986).

Distance between IAF and TNP-ATP

The absorption spectrum of TNP-ATP overlaps the emission spectrum of IAF; thus, TNP-ATP can be an acceptor for energy transfer from IAF. Although the excitation and emission wavelengths of IAF and TNP-ATP are similar, the quantum yield of TNP-ATP is very low compared to that of IAF, so that the contribution of TNP-ATP to the total emission is negligible. Thus, it was possible to measure energy transfer between IAF and TNP-ATP from the quenching of IAF fluorescence by TNP-ATP. Sensitized emission, however, could not be measured because of the low quantum yield of TNP-ATP.

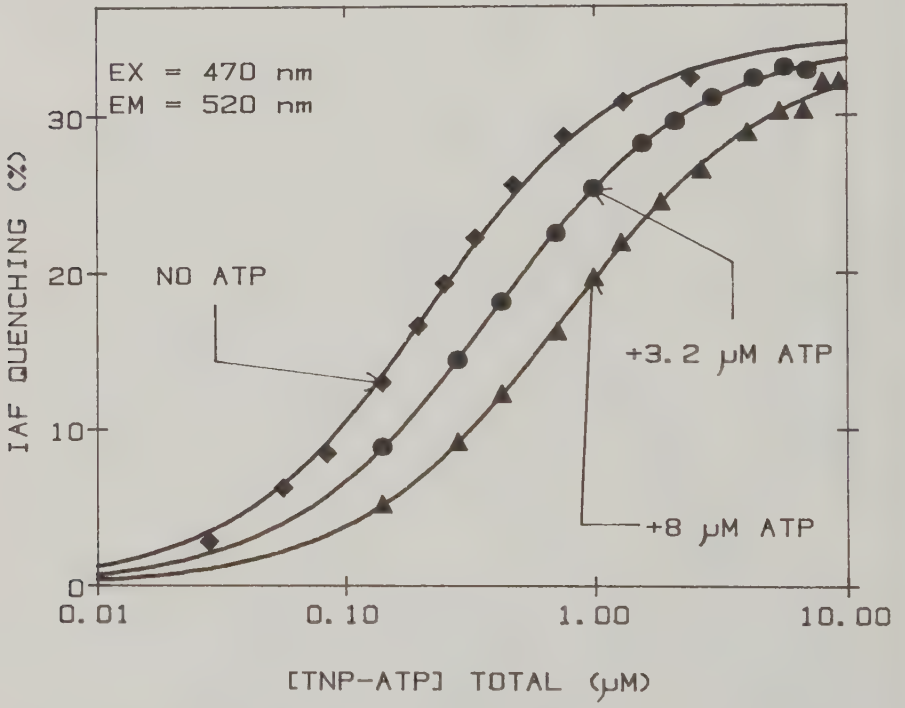

Figure 2. Effect of ATP on quenching of IAF fluorescence by TNP-ATP. Conditions: 50 mM imidazole, 10 mM EDTA, pH 7.5, 102 nM IAF-labelled (Na,K)-ATPase (AO site concentration), and the indicated concentrations of ATP and TNP-ATP. Temperature: 37°C. The solid lines were the best fits of the maximum quenching at saturation and the K_D for TNP-ATP binding to a homogeneous site. The data were corrected for inner filter effects by TNP-ATP.

Fluorescence titrations (Fig. 2) showed that TNP-ATP quenched IAF fluorescence. The concentration dependence of the quenching followed a saturation curve that could be described quantitatively by the binding equation to a homogeneous site using the AO binding capacity for the number of binding sites. The derived values for the dissociation constant of TNP-ATP were: 43 $\pm$ 18 nM (24°C) and 151 $\pm$ 13 nM (37°C) in the absence of ligands of the enzyme; the K_D increased to 5.2 µM in the presence of 100 mM potassium and to 2.9 µM in the presence of ouabain, magnesium and phosphate (data not shown). ATP (1 mM) prevented or reversed the quenching of IAF by TNP-ATP. At µM concentrations, ATP increased the K_D for TNP-ATP (Fig. 2), indicating competition between ATP and TNP-ATP binding. The derived K_D values for ATP were: 345 $\pm$ 45 nM (24°C) and 1-2 µM (37°C). These results indicate that the observed quenching was the result of energy transfer from IAF to TNP-ATP bound to the ATP site of the enzyme.

The values of the maximum quenching at saturating TNP-ATP concentrations ranged from 26% $\pm$ 2% to 37% $\pm$ 2% in preparations with 2.1 and 3 nmol AO sites per mg protein. Those with higher specific activities and AO binding capacities showed higher quenching. Since IAF reacted with all the α-subunits in the preparation, but only a fraction of those α-subunits were able to bind TNP-ATP (presumably because the remainder were inactive or denatured), the measured IAF quenching must have underestimated the actual quenching, because those α-subunits that were unable to bind TNP-ATP would not have been quenched. The fraction of sites that could bind TNP-ATP was calculated from the ratio of the AO binding sites to the IAF-labelled sites. Then, the observed maximum quenching at saturating TNP-ATP was divided by the fraction of sites that could bind TNP-ATP in the preparation to obtain the maximum quenching by bound TNP-ATP.

The maximum IAF quenching at saturating TNP-ATP, corrected for the fraction of functional sites, was 86% in all the preparations, regardless of their fraction of functional sites (the AO binding capacities ranged from 2.1 to 3 nmoles/mg protein). This value agreed with measurements of the lifetime of IAF which showed an increase in short lifetime components (less than 1 ns) in the presence of TNP-ATP, consistent with heterogeneous populations of IAF molecules, some highly quenched and some unquenched by TNP-ATP.

The distance between the IAF site and the ATP site was estimated to be 24 Å from the maximum quenching at saturating TNP-ATP and the distance for 50% energy transfer of 32 Å, calculated from the spectroscopic parameters of IAF and TNP-ATP and the assumption of random orientations between these probes. The uncertainty in this estimate, due to the assumption of random orientations, is not considered to be larger than 20% because both probes exhibit rapid motions as evidenced by their fluorescence anisotropies. The calculation of the distance between IAF and TNP-ATP assumed that the TNP-ATP sites were equal to the AO sites, to correct for the fraction of IAF-labelled sites that could bind TNP-ATP. This assumption has been corroborated experimentally (Moczydlowski and Fortes, 1981a, Fortes, 1986).

The distance between AO and IAF (50 to 56 Å) is smaller than the distance of 64 to 78 Å between AO and TNP-ATP or fluorescein isothiocyanate (FITC) bound to the ATP site, also determined by energy transfer (Jesaitis and Fortes, 1980, Fortes, et al., 1981, Carilli, et al., 1982). In fact, the sum of the distances between IAF and the AO and ATP sites is approximately equal to the distance between the AO site and the ATP site. This suggests that the IAF-reactive site is located in the cytoplasmic region of the α-subunit, closer to the bilayer than the ATP site, and may lie in the line that connects the ATP site with the ouabain site. Since conformational changes are transmitted between the ATP and ouabain sites, the location of IAF between these sites is consistent with the sensitivity of IAF fluorescence to conformational changes of the enzyme.

Implications of the energy transfer between IAF and TNP-ATP

Fortes and Han (1985) have shown that (Na,K)-ATPase from dog kidney is inhibited by reaction of one molecule of FITC per AO site and hence, that each α-subunit functions independently, regardless of the functional state of adjacent α-subunits. They found that TNP-ATP, at concentrations up to 20 μM (about 200 to 400 times higher than the dissociation constant for TNP-ATP), was unable to quench the fluorescence of FITC. They interpreted the absence of energy transfer from FITC to TNP-ATP as evidence that TNP-ATP cannot bind to FITC-labelled (Na,K)-ATPase, because FITC reacts at the TNP-ATP site and prevents TNP-ATP binding.

Since TNP-ATP inhibits (Na,K)-ATPase activity by competing with ATP at both the kinetically defined "high affinity" and "low affinity" ATP "sites" (Moczydlowski and Fortes, 1981a,b), but FITC prevents TNP-ATP binding at both "sites", Fortes and Han (1985) further concluded that (Na,K)-ATPase has a single functional ATP site that changes affinity for ATP during turnover. This conclusion depended on the assumption that the lack of quenching of FITC fluorescence was due to inability to bind TNP-ATP, and that energy transfer from FITC to TNP-ATP should be observed if both probes are close enough to each other. The present finding that TNP-ATP quenched IAF fluorescence by binding to the ATP site demonstrates that TNP-ATP is a good acceptor for energy transfer from fluorescein, and supports the previous conclusion that (Na,K)-ATPase has a single functional ATP site (Moczydlowski and Fortes, 1981a,b, Fortes and Han, 1985).

CONCLUSIONS

1. IAF labels an iodoacetate-reactive site of the α-subunit, probably a cys residue within the 18 kDa segment between the trypsin and chymotrypsin cleavage sites.
2. The IAF site is 50 to 56 Å away from the ouabain site, and about 24 Å away from the ATP site.
3. The IAF site is located in the cytoplasmic portion of the α-subunit, near the bilayer, and may lie within the line that connects the ATP site with the ouabain site.
4. The demonstration of energy transfer from IAF to TNP-ATP, together with the lack of energy transfer from FITC to TNP-ATP, supports the conclusion that (Na,K)-ATPase has a single ATP site that changes affinity during turnover, rather than two different ATP sites.

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HIGH-PERFORMANCE GEL CHROMATOGRAPHY OF ACTIVE SOLUBILIZED Na^+,K^+-ATPase MAINTAINED BY EXOGENOUS PHOSPHATIDYLSERINE

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INTRODUCTION

Na^+,K^+-ATPase is mainly composed of two kinds of polypeptide, referred to as α and β. We have shown previously by means of low-angle laser light scattering (LALLS) photometry coupled with high-performance gel chromatography (HPGC) that the major molecular species of the solubilized ATPase are those in the form αβ and $(\alpha\beta)_2$ (Hayashi et al., 1983, 1985). It is, however, not yet made clear which of the two oligomeric forms of αβ-protomer and $(\alpha\beta)_2$-diprotomer is the minimum functional unit for hydrolysis of ATP. To make clear this point, we believe that molecular weight of the solubilized enzyme exhibiting ATPase reaction should be measured by the HPGC/LALLS method. Thus, the HPGC procedure has been designed in this study so that the ATPase turns over during its passage through the column at 20°C. This has been accomplished by the addition of an exogenous phospholipid, phosphatidylserine (PS) to the elution buffer, so that the solubilized enzyme constantly exhibits the ATPase activity comparable to that of the membrane-bound enzyme during the chromatography.

METHODS

The membrane-bound Na^+,K^+-ATPase was purified from dog kidney and the activity was 37.2 ± 1.3 μmol Pi min^{-1} mg^{-1} protein under optimal conditions at 37°C. The membrane-bound enzyme was solubilized with octaethylene glycol n-dodecylether ($C_{12}E_8$) as described previously (Hayashi et al., 1985). In some cases, prior to the solubilization, the

membrane-bound enzyme (3.1 mg ml^{-1} protein) was incubated with 1.5 mM ouabain in a solution of 3.1 mM $MgCl_2$, 3.1 mM dithioerythritol, 1.5 mM EDTA, 15 % glycerol, 20 mM imidazole and 16 mM Hepes at pH 7.0 and 37°C. After incubation for 10 min, the solution was cooled to 0°C, and KCl and then $C_{12}E_8$ were added to the solution so that the final composition became the same as that for the untreated enzyme with respect to the constituents other than ouabain and $MgCl_2$.

The solubilized enzyme was chromatographed on a TSK-gel G3000SW column (7.5 x 600mm) equipped with a guard column (TSK-guard column SW, 7.5 x 75mm). The elution buffer was made to contain the same components as those in an ATPase assay medium, i.e., 4 mM ATP, 0.05 M NaCl, 0.05 M KCl, 4 mM $MgCl_2$, 1 mM EDTA, 10 mM imidazole and 15 mM Hepes as well as 0.2 mg ml^{-1} $C_{12}E_8$ at pH 7.0, unless otherwise stated. Phosphatidylserine from bovine brain with 98 % purity (Sigma Chemical Co.) was added to the elution buffer as follows : An aliquot of the chloroform-methanol solution containing 7.5 to 22.5 mg of PS was evaporated to dryness in a stream of nitrogen. Several ml of the elution buffer was added to the residue of PS and sonicated until visible fragments of PS disappeared, leaving a slightly cloudy solution. The resultant suspension was poured into a large volume of the elution buffer without PS so that the concentration of PS became 15 to 60 µg ml^{-1}. The resultant solution became clear after incubation overnight at room temperature, and was used as the elution buffer. The column as described above had been equilibrated with about 100 ml of the elution buffer thus prepared at a flow rate of 0.50 ml min^{-1} and 20°C. An aliquot of 230 µl containing 0.39 mg protein of the solubilized enzyme was charged onto the columns, and the columns were eluted with the elution buffer. The eluate from the columns was monitored with a differential refractometer (RI ; TSK model RI-8000). One ml fractions of the eluate were collected in test tubes preloaded with 0.5 ml of 5 % SDS to stop the enzyme reaction. The concentration of Pi in each fraction was plotted against the retention time at which the fraction was taken (see Fig. 1).

Specific ATPase activity of the enzyme exhibited in the column was calculated from the amount of Pi liberated during the passage through the columns, the time taken to liberate the amount of Pi, and the protein amount eluted. The protein amount was estimated from the area under the peak of RI

using the ratio of differential refractive index to protein concentration, that is, specific refractive index increment.

RESULTS & DISCUSSION

Immediately after solubilization with $C_{12}E_8$, Na^+,K^+-ATPase was charged onto a TSK-gel G3000SW column equilibrated with the elution buffer containing the components necessary to hydrolyze ATP, but not containing any exogenous phospholipid, and the column was eluted with the same elu-

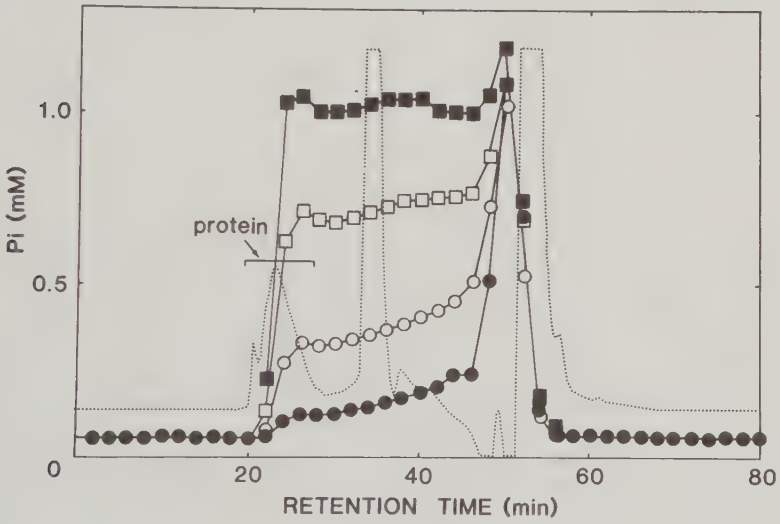

Figure 1. ATP hydrolysis by the solubilized Na^+,K^+-ATPase during the high-performance gel chromatography and the effect of exogenous phosphatidylserine on it. The solubilized enzyme was charged onto the HPGC column equilibrated with the elution buffer containing 0 (●), 15 (○), 30 (□) or 45 $\mu g\ ml^{-1}$ (■) of PS, and the column was eluted with the elution buffer. The concentrations of Pi in fractions of eluate were plotted against the retention time at which the fractions were taken. The dotted line shows the pattern of differential refractive index obtained when no PS was included in the elution buffer. The peaks other than those indicated by "protein" were non-protein components (Hayashi et al., 1983).

tion buffer at 20°C. The enzyme protein was eluted as a major peak, and the other large peaks were attributable to non-protein components. The Pi liberation did not occur constantly while the enzyme passed through the column. Judging from the elution pattern of Pi, the enzyme was very active in the initial phase of the passage through the column (see the data obtained at the retention time of around 50 min and without exogenous phospholipid in Fig. 1), but in the final phase the activity became less than about 6 % of the maximum activity observed in the initial phase. The solubilized enzyme was applied to the column equilibrated with the elution buffer containing exogenous PS, and the column was eluted with the same elution buffer. As the concentration of PS was increased from 0 to 45 $\mu g\ ml^{-1}$, the amount of Pi liberated in the final phase increased while that liberated in the initial phase was almost constant. The rate of Pi liberation in the final phase became almost the same as that in the initial phase by the addition of 45 $\mu g\ ml^{-1}$ PS to the elution buffer (Fig. 1).

The major protein component of the solubilized enzyme was isolated by the chromatography with the elution buffer containing neither PS nor ATP and otherwise the same constituents as those described in METHODS. The amount of endogenous phospholipid bound to the protein component eluted was estimated to be 5.8 ± 1.2 mol phospholipid per 1.5×10^5 g protein (n=3) by the ultramicro method of Bartlett (1959), and found to be far less than that (40 to 50 mol per 1.5×10^5 g protein) required for the solubilized enzyme to exhibit the maximum activity (Ottolenghi, 1979). Thus, it can be probably concluded that the protein component can hydrolyze ATP very fast in the initial phase of chromatography since endogenous phospholipids still bind sufficiently to it even in the absence of exogenous PS, but that the elimination of the endogenous phospholipids that occurred in the later phase make the enzyme inactive. Accordingly, exogenous PS, when included in the elution buffer, may be substituted for the endogenous phospholipids, and enable the enzyme to exhibit high ATPase activity in the whole phase of the chromatography.

The specific ATPase activity of the solubilized enzyme exhibited during passage through the column was estimated with the elution buffer containing 60 $\mu g\ ml^{-1}$ PS and otherwise the same constituents as those described above. The amounts of the enzyme protein charged onto the column were

varied between 4.5 and 182 µg protein. To estimate the ATPase activity depending on only the exogenous phospholipid, the rate of Pi-liberation was calculated using the amount of Pi detected in the eluate at the retention time of earlier than 43 min (see Fig. 1). As shown by open circle in Fig. 2, the resultant activities were not dependent on the protein amount charged onto the column, but constant at the value of 2.64 ± 0.08 µmol Pi min^{-1} mg^{-1} protein (n=5). The membrane-bound Na^+,K^+-ATPase from which the solubilized enzyme was prepared was assayed for ATPase activity in the absence of PS and $C_{12}E_8$ under otherwise the same conditions as those in the column. The activity was 3.34 ± 0.17 µmol Pi min^{-1} mg^{-1} protein (n=3), showing that the activity of the solubilized enzyme in the column was equivalent to 79 %

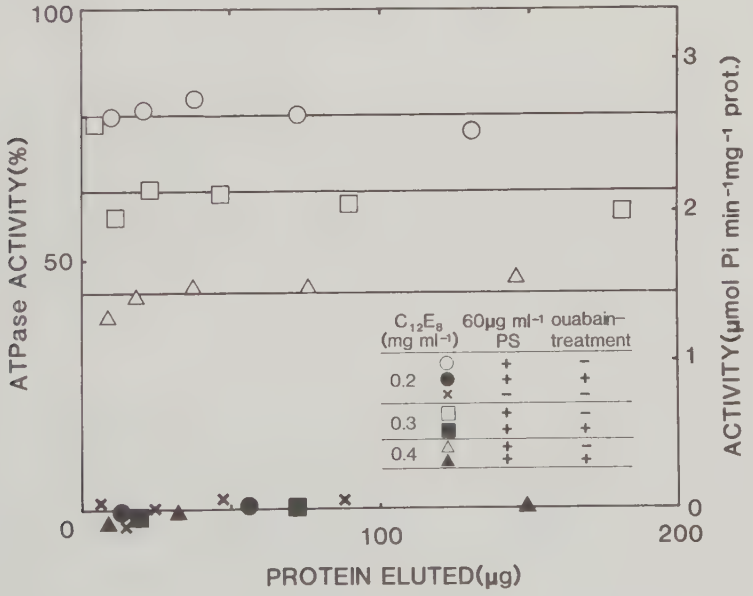

Figure 2. Specific ATPase activities of the solubilized enzyme estimated during the HPGC carried out with various amounts of protein charged in the presence of various concentrations of $C_{12}E_8$ at 20°C. One hundred % (= 3.34 ± 0.17 µmol Pi min^{-1} mg^{-1} protein) corresponds to the activity of the membrane-bound enzyme estimated in the absence of $C_{12}E_8$ and PS under otherwise the same conditions as those in the column.

of that of the membrane-bound enzyme. When the concentration of $C_{12}E_8$ was increased from 0.2 to 0.3 and 0.4 mg ml^{-1} keeping the concentration of PS constant at 60 μg ml^{-1}, the activities of the solubilized enzyme in the column were decreased to 64 and 43% of that of the membrane-bound enzyme, respectively (Fig. 2). The results strongly suggested that PS competed with $C_{12}E_8$ for hydrophobic regions of the enzyme, and that the higher retention of PS to the enzyme occurred in the lower concentration of $C_{12}E_8$ and brought about the higher ATPase activity in the column.

When the membrane-bound enzyme was treated with ouabain and then solubilized, Pi liberation with the solubilized enzyme thus obtained could not be observed at all during the chromatography even in the presence of 60 μg ml^{-1} PS regardless of the concentrations of $C_{12}E_8$ (Fig. 2). Evidently the ATPase activity maintained by the exogenous PS is to be coupled to cation-transport of Na^+ and K^+.

The summary is as follows : The solubilized Na^+,K^+-ATPase proved to be almost inactive in the HPGC column eluted with the elution buffer which ingredients were the same as those in an ATPase assay medium. On the contrary, the enzyme could exhibit the activity comparable to that of the membrane-bound enzyme by the addition of 60 μg ml^{-1} exogenous PS to the elution buffer. The activity was sensitive to ouabain.

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PROPERTIES OF THE Na^+, K^+, AND ATP BINDING SITES OF THE OUABAIN-COMPLEXED (Na^++K^+)-ATPase: IMPLICATIONS FOR THE MECHANISM OF OUABAIN ACTION

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INTRODUCTION

To know the mechanism of an inhibitor's action on an enzyme or a carrier, we need to know how the inhibitor affects substrate interactions with the protein. The substrates of (Na^++K^+)-ATPase (the sodium pump) are Na^+, K^+, and ATP. How does ouabain, the classical inhibitor of the pump, affect the properties of binding sites for these substrates? It is surprising how little attention has been paid to this question. The few studies that provide some pertinent information include those suggesting the exclusion of all three substrates by ouabain (Hansen et al, 1971; Matsui and Homareda, 1982), and those showing the bindings of all to the ouabain-complexed enzyme (Forbush, 1983; and references therein). To resolve the uncertainties, and to fill the existing gaps, we have taken advantage of ouabain's slow release from the enzyme, and we have used the following approach to determine the affinities, the numbers, and the interactions of the substrate sites of the ouabain-complexed enzyme.

Assume that substrate S_1 binds to the enzyme-ouabain complex in rapid equilibrium fashion and alters the rate of unidirectional release of ouabain as follows:

$$O + E \xleftarrow{k_1} EO + S_1 \rightleftharpoons S_1EO \xrightarrow{k_2} S_1E + O$$

If k is the measured rate constant for ouabain release at any $[S_1]$, it can be shown that the plot of $k - k_1$ (or

$k_1 - k$) as a function of $[S_1]$ is hyperbolic, and that K_d may be obtained from this plot or any of its usual variations; e.g., double-reciprocal, Scatchard, or Hill plots. If the experimentally determined plot is indeed hyperbolic ($n=1$), the number of S_1 sites per ouabain binding site can not be determined (since there may be multiple but equivalent S_1 sites), but n values clearly different from one would indicate multiple interacting S_1 sites. A more obvious and certain indication for multiple S_1 sites (with or without interactions) would be a biphasic plot; i.e., if with increasing $[S_1]$ there is first an increase and then a decrease in k, or vice versa. (It is important to note that such a phenomenon cannot be explained by two interconverting forms of EO, each having one site but different affinities for S_1.) When two or three substrates are introduced in the above scheme, the resulting equations are analogous to those applicable to bi- and terreactant systems of enzyme kinetics with rapid equilibrium assumptions. The relationship between S_1, S_2, and S_3 sites may be determined by the conventional graphical analyses used for multiple substrates, or activators, or inhibitors.

RESULTS

To study the effects of ouabain on the substrate sites, the two classical ouabain-enzyme complexes (Hansen, 1984) were used. Complexes I and II were prepared in the presence of Na^++MgATP and MgPi respectively, using saturating concentrations of [^{3}H]ouabain and the purified canine kidney enzyme. The complexes were washed in cold, suspended in an EDTA-containing solution and allowed to dissociate at 37°C (Kakar *et al*, 1985). As expected, both complexes dissociated exponentially; complex I with the rate constant of about 0.05 min^{-1}, and complex II with the rate constant of about 0.004 min^{-1}. The effects of varying concentrations of Na^+, K^+, and ATP (each ligand separately, and in combination with others) were then studied on the two complexes; and the data were analyzed by the approach discussed above to characterize the binding sites of the complexes. A limited portion of the results of these studies concerning the ATP sites of complex I have been reported before (Kakar *et al*, 1985). The details of the remaining data will be published elsewhere. Here we summarize those aspects of our findings that are most pertinent to the mechanism of ouabain effect on the enzyme.

Substrate Sites of Complex I.

As expected from previous findings (Forbush, 1983; Hansen, 1984), either Na^+ or K^+ slowed the rate of release of ouabain from complex I. Experiments of Fig. 1 showed that $K_{0.5}$ of K^+ was about 0.1 mM, and $K_{0.5}$ of Na^+ was about 1.3 mM (n values were not significantly different from one). The affinities of these Na^+ and K^+ sites of complex I do not seem to be significantly different from the high-affinity Na^+ and K^+ sites of the native enzyme (Jensen et al, 1984). When the combined effects of Na^+ and K^+ were examined (data not shown), the two seemed to be mutually exclusive inhibitors of ouabain release. The presence of two coexisting ATP sites on complex I was established before (Kakar et al, 1985). Because of the well-known effects of ATP on the apparent affinities of the native enzyme for Na^+ and K^+, it was of interest to examine the relation of Na^+, K^+, and ATP sites in complex I. Appropriate experiments showed, however, that ATP could not overcome the inhibitory effects of Na^+ and K^+ on ouabain release, and that the $K_{0.5}$ values of the two ions were the same in the presence and absence of ATP. Evidently, either Na^+ and ATP, or K^+ and ATP, may bind simultaneously, and the binding of one has no effect on the other's affinity.

Substrate Sites of Complex II.

As shown in Fig. 2, Na^+ stimulated ouabain release with $K_{0.5}$ of 600 mM (n=2), indicating the presence of a set of low-affinity Na^+ sites. K^+ had no effect on ouabain release when used alone (Fig. 2); but it blocked the effect of Na^+ with $K_{0.5}$ of about 200 mM (data not shown) indicating the existence of low-affinity K^+ sites on complex II. Conventional experiments on the combined effects of Na^+ and K^+ (data not shown) indicated that the two ions could bind simultaneously to their low affinity sites, and that the binding of one had no effect on the affinity of the other. ATP had no effect on ouabain release when used alone or in combination with K^+; however, ATP was an allosteric regulator of the low-affinity Na^+ sites (Fig. 2), lowering the $K_{0.5}$ of Na^+ to 200 mM (n=1). $K_{0.5}$ for this effect of ATP was 0.1 mM.

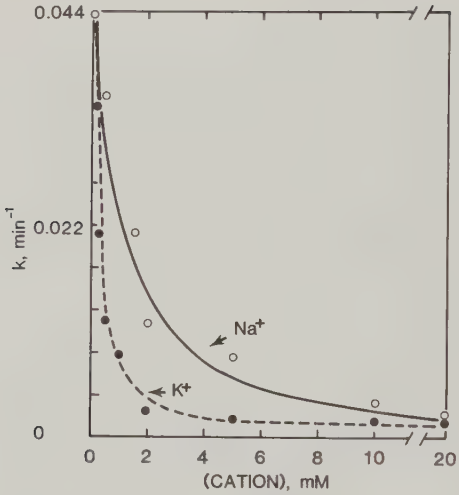

Fig. 1. Effects of varying concentrations of Na^+ and K^+ on the rate of release of ouabain from complex I.

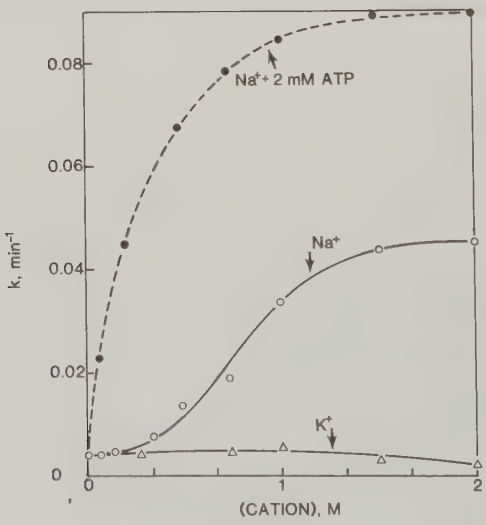

Fig. 2. Effects of varying concentrations of Na^+ and K^+ on the rate of release of ouabain from complex II.

Relation of Complex I to Complex II.

To test the previously suggested possibility that the two complexes may be interconvertible (Hansen, 1984), we did a series of experiments involving both complexes and with designs similar to the following example: Complex II was destabilized in the presence of Na^++ATP. As shown in Fig. 2, the dissociation rate of complex II under these conditions may become as great as that of complex I. When complex II was about 50% dissociated, it was cooled and washed. When the remaining complex was exposed to Na^+, K^+, and ATP, the properties of the substrate sites were precisely the same as those of the original complex II rather than those of complex I. The same pattern was observed in all experiments of this series, indicating that the two complexes are not interconvertible.

We confirmed the observation of several laboratories by showing that the maximal level of bound ouabain was the same in both complexes, and equal to maximal phosphorylation capacity of the enzyme. We also established that when one complex was formed in the presence of saturating ouabain and washed, its exposure to ouabain under conditions used for the formation of the other complex did not result in any additional ouabain binding.

DISCUSSION

Our studies do not provide any evidence in support of the interconvertibility of complexes I and II. To explain the drastically different properties of the substrate sites on two noninterconvertible complexes, two alternatives may be offered: First, ouabain may freeze the enzyme in two different conformations as it has been suggested before (Hansen, 1984). The second possibility, which has not been considered before, is that there are two dissimilar but mutually exclusive binding sites for ouabain. We prefer this alternative because it can be tested experimentally. Regardless of why the two complexes are different, however, the present results lead to the following observations concerning the mechanism of ouabain interaction with the enzyme:

1. It is well-established that ouabain has higher affinity for phosphorylated states of the enzyme than for

the dephosphorylated states (Askari et al, 1983; Hansen, 1984). Citation of this fact, however, is often accompanied by the unjustified assumption that the phosphoenzyme obtained in the presence of MgPi is the same as the E_2P that is obtained in the presence of MgATP and physiological concentrations of Na^+ (e.g. Fortes and Lee, 1984; Robinson and Flashner, 1979). Many years ago Post et al (1975) showed that this is not true; and the present findings emphasize again that we should not confuse the phosphoenzyme obtained from Pi with either E_2P or E_1P. See also Yoda and Yoda (1987; and this conference) on the uncertainties about the relative reactivities of various phosphoenzymes toward ouabain.

2. Although the two complexes are obtained through ouabain binding to phosphoenzymes, as shown before (Hansen, 1984) it is the dephosphorylated complex in each case whose substrate sites is being studied. It is clear from the present results that the ouabain-complexed dephosphoenzyme (at least within one collection of its various states) does alternate between a high-affinity K^+-binding form and a high-affinity Na^+-binding form. What then are we to do with the often-repeated statements that ouabain "favors the E_2 state" or "stabilizes the enzyme in the E_2 state"? We should stop repeating them.

3. The high-affinity Na^+ and K^+ sites exist in complex I, but are not detected in complex II. The undetectability of these sites does not indicate their absence, since the sites would not be detected if their occupancy did not affect the ouabain site. In fact some of our results, not presented here, indicate that under certain conditions some high-affinity sites may be unmasked in complex II. In any case, to reconcile the present findings with the radioligand binding experiments in which ouabain has been found to inhibit high-affinity Na^+ and K^+ bindings to the enzyme (Matsui and Homareda, 1982), we have to assume one of the following: Either ouabain inhibits some, but not all, of the high-affinity cation sites, or that ouabain inhibits binding to these sites in some, but not all, states of the enzyme. If the latter is the case, since at this time we cannot be certain what states are induced when the operating pump is exposed to ouabain, we should keep an open mind about the status of the high-affinity transport sites of the ouabain-inhibited pump.

4. The nature of the low-affinity Na^+ and K^+ sites of complex II is not clear (See Askari, this conference). If we assume, as it is generally done, that these sites are altered states of the high-affinity sites, demonstration of their coexistence in complex II would indicate that the enzyme has physically distinct Na^+ and K^+ sites, and that ouabain alters interactions between these sites. Put in other words, Na^+ and K^+ sites that seem to be mutually exclusive in some experiments with the native enzyme (and in complex I) are no longer so in complex II. The same type of phenomenon is also evident in relation to K^+ and ATP sites. The usual antagonism between the two in the native enzyme is abolished upon ouabain binding. This ability of ouabain to disrupt site-site interactions of the native enzyme may have more to do with its inhibitory effect on the pump than ouabain-induced affinity changes or blockade of substrate sites.

CONCLUSIONS

We started by asking a simple question: How does inhibitor binding affect substrate binding? We have learned that there is no simple answer, and that we are far from understanding the complexities of the mechanism of ouabain inhibition of the pump. Because so much work on ouabain interaction with the enzyme has been done for so long, there is a tendency to assume that what needs to be known about the nature of the interaction is known. This assumption may be excused at times as an indication of harmless optimism, but it could also become seriously misleading. This is especially the case when ouabain is used as a "tool" in experiments that are intended to settle important controversies, such as those on whether or not E_1P and E_2P are the normal intermediates of the pump (Fortes and Lee, 1984), and whether or not Na^+ and K^+ may bind to the pump simultaneously (Matsui and Homareda, 1982). No doubt that in the end ouabain will turn out to be an important and useful tool. After so many years, however, it still looks more like a fascinating Rube Goldberg contraption rather than a tool.

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THE CATALYTIC SUBUNITS COOPERATE IN (Na^++K^+)-ATPase: DEMONSTRATION WITH $MgPO_4$ AND MgATP COMPLEX ANALOGUES -

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INTRODUCTION

Although it has been demonstrated, that the detergent-solubilized monomeric α-subunit of (Na^++K^+)-ATPase has all catalytic properties and occludes Na^+ and K^+ (Vilsen et al., 1987), it is undecided so far, whether a single α-subunit in the membrane-bound enzyme furnishes the minimal requirements to pump Na^+ and K^+ against their electrochemical gradient. The contrasting models of the "single site model" (Smith et al., 1980; Moczydlowski and Fortes, 1981) and of the "flip-flop model of cooperating subunits" (Stein et al., 1973; Repke and Schön, 1973) have been proposed to describe the events during the pumping process. Since all kinetic requirements of ATP hydrolysis are fulfilled by a single catalytic α-subunit, Smith et al. (1980) and Moczydlowski & Fortes (1981) postulate as a minimal model the existance of a "single site model". In this model high and low affinity ATP binding sites are formed by a single α-subunit consecutively during ATP hydrolysis. This means, that during the catalytic process the capacity of the high affinity ATP binding site in the E_1 conformational state decreases to the account of the increase of the capacity of the low affinity ATP binding site when the enzyme converts to the E_2 conformation during (Na^++K^+)-activated ATP hydrolysis. On the contrary, the "flip-flop model" postulates the cooperation of at least two catalytic α-subunits. Each of them works according to the Albers-Post model in a 180° inverse way. In this model the capacities of the high and low affinity ATP binding sites remain constant. A considerable quantity of data has been accumulated to support the latter model

(Askari, 1982). Nevertheless a definitive proof for the validity of the model is lacking.

Recently, we found that the MgATP complex analogue chromium(III)ATP (CrATP) interacts with the high affinity ATP binding site (Pauls et al., 1980) and arrests the turnover of the enzyme in the E_1 conformation by the formation of a stable phosphointermediate. CrATP does not fuel active (Na^++K^+) transport, it supports however the Na^+-Na^+ and also the K^+-K^+ exchange reactions (Pauls et al., 1986). The finding of a CrATP supported K^+-K^+ exchange is somewhat unexpected, because this reaction is considered to occur in the E_2 conformation. The K^+-K^+-exchange under these conditions might occur, however, when (Na^++K^+)-ATPase would act according to the "flip-flop model" as a (α,β)-dimer (Repke and Schön, 1973), wherefrom one (α,β)-monomer is arrested in the E_1 conformation and the other one has still the possibility to be in the E_2 conformation. The recent demonstration that palmitoyl-CoA stimulates (Na^++K^+)-ATPase activity by interaction with the regulatory low affinity ATP binding site (Kakar et al., 1987) not merely further supports the concept of interacting α-subunits. The finding also shows that it is possible to find ATP analogues which exclusively bind to the low affinity ATP binding site. Such analogues are a very helpful tool to test the correctness of the "single site model", because the capacity and the catalytic properties of the high affinity ATP binding site can still be investigated. Cobalt tetrammine ATP (CoATP) which has formerly been shown to inactivate (Na^++K^+)-ATPase (Pauls et al., 1986) is a candidate for a MgATP analogue that might binds to the low affinity ATP binding site like it is the $MgPO_4$ analogue $Co(NH_3)_4PO_4$. This paper confirms this suggestion and shows that both substances can be used to disprove the "single site model" and to demonstrate a cooperation of catalytic subunits.

MATERIALS AND METHODS

All experiments were performed with sheep or pig kidney enzyme of a specific activity of 15-30 µmoles ATP hydrolyzed per min at 37°C. The assays to test the enzyme activity, the capacity of the high affinity ATP binding site and Na^+-dependent formation of the phosphointermediate have been described (Scheiner-Bobis et al., 1987) like the synthesis of $Co(NH_3)_4ATP$ (Cornelius et al., 1977) and $Co(NH_3)_4PO_4$

(Siebert, 1958). Na^+-ATPase activity was determined by a modification of the procedure of Neufeld and Levy (1969). Inactivation of $(Na^+ + K^+)$-ATPase by increasing concentrations of CoATP and $Co(NH_3)_4PO_4$, respectively, occured at 37°C either in 10 Tris-HCl or 70 mM imidazole-HCl pH 7.25.

RESULTS AND DISCUSSION

Incubation of $(Na^+ + K^+)$-ATPase at 37°C with increasing concentrations of $Co(NH_3)_4ATP$ or $Co(NH_3)_4PO_4$ results in a slow inactivation of the enzyme. From the plot of the reciprocal concentration of both substances versus the inactivation half-life the apparent dissociation constants of 500 µM for the MgATP analogue complex and of 300 µM of the $MgPO_4$ complex analogue with the enzyme are calculated (Fig. 1). The inactivation rate constant k_2 at saturating concentrations of CoATP is 0.05 min^{-1} and at saturating concentrations of $Co(NH_3)_4PO_4$ is 0.05 min^{-1} as well (Fig. 1). Both

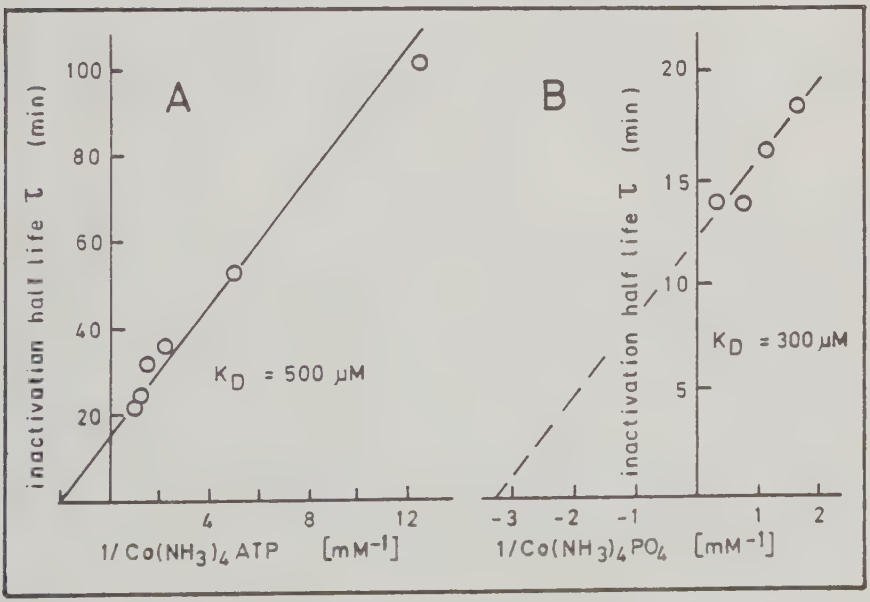

Figure 1: Apparent inactivation half-life of $(Na^+ + K^+)$-ATPase as a function of the reciprocal concentrations of $Co(NH_3)_4ATP$ (A) and $Co(NH_3)_4PO_4$ (B).

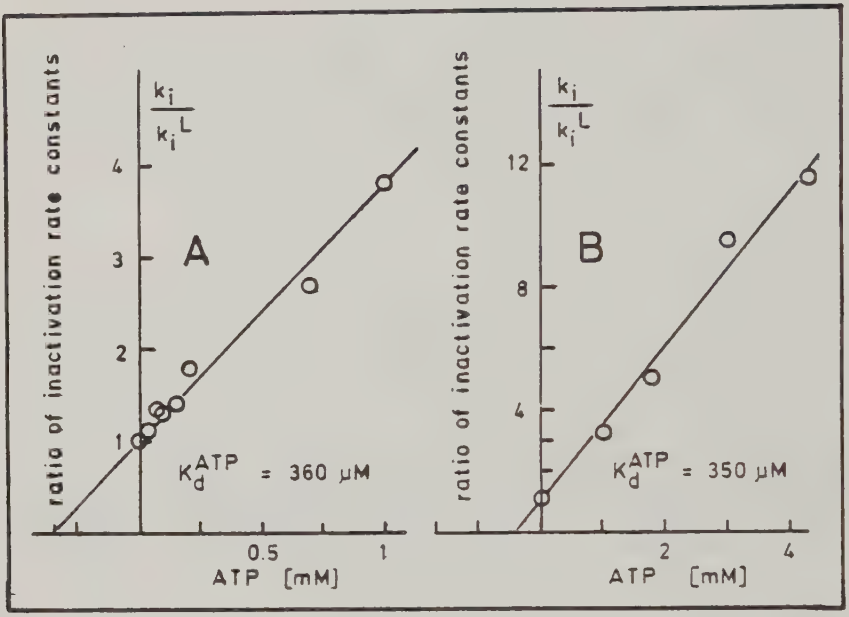

Figure 2: ATP-Protection of $(Na^+ + K^+)$-ATPase against the inactivation by 600 µM $Co(NH_3)_4ATP$ (A) and 600 µM $Co(NH_3)_4PO_4$ (B). Plot according to Eckert et al. (1977).

substances do not inactivate at 0°C. The inactivation of $(Na^+ + K^+)$-ATPase by both substances is prohibited by the presence of ATP. From the Eckert-plot of the ATP-protective effect it is apparent that both substances interact with a site, to which ATP binds with the dissociation constant of the enzyme ATP complex of K_d = 360 µM (Fig. 2). As could be shown by the use of [γ-^{32}P]- and [α-^{32}P]-labelled CoATP, concomitantly with the inactivation processes an incorporation of the radioactivity occurs into the α-subunit (not shown). The CoATP-inactivated enzyme incorporates radioactive ouabain in the absence of any additional ligands of the enzyme. This ist not the case with a control enzyme (Scheiner-Bobis et al., 1987).

The above findings show that the MgATP complex analogue $Co(NH_3)_4ATP$ (CoATP) and $MgPO_4$ complex analogue $Co(NH_3)_4PO_4$ ($CoPO_4$) interact both with the low affinity ATP binding site of the enzyme (E_2), wherefrom a stable cobalt tetrammine phosphoenzyme is formed (eqn 1,2):

$$E_2 + CoATP \rightleftharpoons E_2 \cdot CoATP \xrightarrow{k_2} E_2 \overset{P}{\underset{ADP}{\cdots}} Co \qquad (1)$$

$K_d = 500\ \mu M$

$$E_2 + CoPO_4 \rightleftharpoons E_2 \cdot CoPO_4 \xrightarrow{k_2} E_2 \overset{P}{\cdots} Co \qquad (2)$$

$K_d = 300\ \mu M$

The inactive phosphoenzyme is apparently in a E_2-like conformational state, since it forms easily the ouabain enzyme complex (Scheiner-Bobis et al., 1987), which is assumed to exist in the E_2 conformation.

The specific modification of the low affinity ATP binding site by a MgATP and a $MgPO_4$ complex analogue opens up the possibility to study the properties of the high affinity ATP binding site. According to the "single site model" (Smith et al., 1980; Moczydlowski & Fortes, 1981) the capacity of the high affinity ATP binding sites and the capability to form Na^+-dependently a phosphorylated intermediate

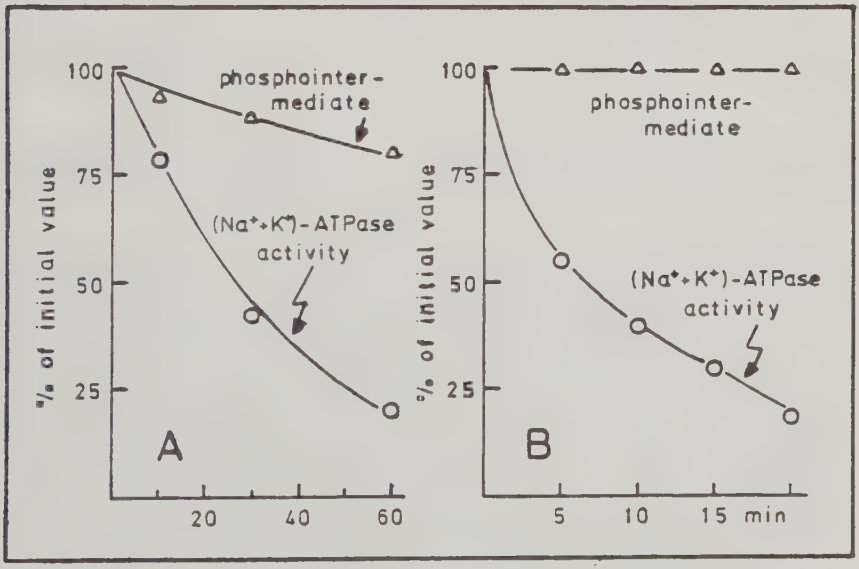

Figure 3: Comparision on the effects of 500 µM $Co(NH_3)_4ATP$ (A) and of 600 µM $Co(NH_3)_4PO_4$ (B) on the activity of $(Na^+ + K^+)$-ATPase and on the Na^+-dependent phosphorylation of the enzyme protein from [γ-^{32}P]ATP.

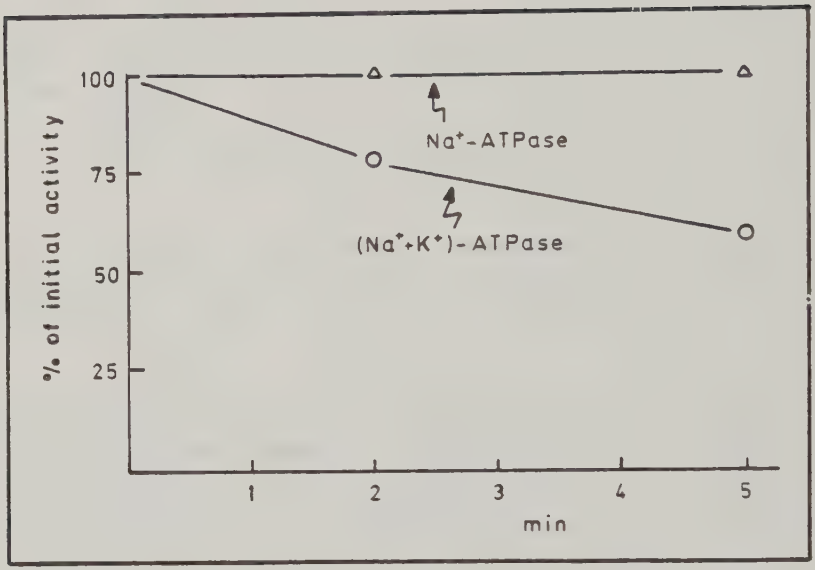

Figure 4: Effect of 600 µM $Co(NH_3)_4PO_4$ at 37°C on the activity of (Na^++K^+)-ATPase (22.7 U/mg) and of Na^+-ATPase (0.12 U/mg).

should decrease when the amount of the E_2ATP complex or of the E_2 phosphoenzyme increases.

In contrast to the single site model, a 70% inactivation of (Na^++K^+)-ATPase by incubation with 500 µM CoATP let the capacity of the high affinity ATP binding site unaffected (Scheiner-Bobis et al., 1987), just as the capability of the inactivated enzyme to phosphorylate Na^+-dependently from the high affinity ATP binding site the enzyme (Fig. 3A). Likewise, a 82% inactivation of (Na^++K^+)-ATPase by 600 µM $Co(NH_3)_4PO_4$ did not affect the capacity of the Na^+-dependent phosphointermediate (Fig. 3B). Moreover, inactivation of (Na^++K^+)-ATPase by 600 µM $Co(NH_3)_4PO_4$ did not affect the activity of the Na^+-ATPase (Fig. 4). This enzyme activity is considered to mirror the Na^+-dependent aspartyl phosphorylation of the enzyme from the high affinity ATP binding site and its Na^+-activated hydrolysis in the E_1 conformation.

The peculiar finding, that the formation of a stable E_2 phosphointermediate from $Co(NH_3)_4ATP$ and $Co(NH_3)_4PO_4$ leads

to the inactivation of $(Na^+ + K^+)$-ATPase, is consistent with the Albers-Post scheme of $Na^+ + K^+$ activated ATP hydrolysis. Inconsistent with the "single site model", is the finding that the capacity of the high affinity ATP binding site, the Na^+-dependently formed E_1 phosphointermediate and the Na^+-ATPase activity does not decrease, when the capacity of the E_2 phosphointermediate increases. The most likely interpretation of this finding is, that the "single site model" is not valid. Apparently we look upon an other α-subunit than the $Co(NH_3)_4PO_4$- and $Co(NH_3)_4ATP$-modified one, when we measure the capacity of the high affinity ATP binding site, the E_1 phosphointermediate and the Na^+-ATPase activity. Since, however, $(Na^+ + K^+)$-ATPase is inactivated by the fixation of one α-subunit in the E_2 state we have to postulate that at least 2 catalytic subunits have to cooperate in the pumping process and that consistent with the Albers-Post scheme the α-subunit in the E_2 conformation is reconverted into the E_1 conformation. Such a model of cooperating subunits may work in a flip-flop like manner (Repke and Schön, 1973) or differently (Plesner, 1987). The precise way of interaction has still to be worked out.

ACKNOWLEDGEMENT

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Conformational Transition; Ion Occlusion and Phosphointermediates

The Na^+,K^+-Pump, Part A: Molecular Aspects, pages 229-248
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OVERVIEW:

OCCLUDED IONS AND Na,K-ATPase

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WHAT ARE OCCLUDED IONS?.

Ion transport proteins such as Na,K-ATPase bind ions on one side of a biological membrane, translocate them across the membrane, and release them on the other side. This article is concerned with states which occur during the translocation process in which the ions are hidden within the interior of the membrane protein, inaccessible to the solutions on both sides. Such states are termed "occluded states", and while the ions are unable to reach the solutions on either side they are termed "occluded ions". The existence of occluded states has been conjectured from kinetic evidence -- the protein structure comprising the binding site is still entirely unknown, as is the nature of the barriers preventing access of the ions to the medium.

Operationally, ion occlusion is usually demonstrated as the binding of radiolabelled ions to Na,K-ATPase, under conditions in which the unbound ions are removed from the medium by ion exchange (Beaugé *et al.*, 1979) or the sample is filtered to remove exchangeable ions (Forbush, 1984b). Alternatively, occlusion has been inferred from less direct measurements in which the behavior of Na,K-ATPase is seen to be affected by prior exposure to a cation (Post *et al.*, 1972). As will be discussed below, the evidence is quite strong that, in the case of Na and K, these ions are bound to transport sites, supported by excellent agreement between the behavior of the pump in transport reactions and the behavior of the ions in occluded states. Divalent ions such as Mg and Ca may also be bound to the Na,K-ATPase,

probably coordinated with the phosphate moiety in the phosphorylated intermediate -- although these ions appear to be bound in such a way that they are inexchangeable with the medium (Fukushima and Post, 1978), they are probably not bound at transport sites, and they are generally not termed "occluded" ions.

The rate of dissociation of an occluded ion from its binding site is very low (0.001 - 100 s^{-1}) compared to the rate of dissociation of an ion from a binding site exposed to an aqueous medium and limited only by diffusion (~100,000 s^{-1}, for K_d = 0.1mM). While we are apt to refer to these ions as "tightly bound", "tight binding" does not necessarily imply a high affinity for the ion. Since *access* to the site is restricted in the occluded conformational state, the rate of entry of an ion into the occlusion site is also low, and the equilibrium binding constant for the occluded ion may be similar to the binding constant when the site is exposed to the medium. In some cases, occupancy of the transport sites does favor formation of the occluded conformation and the effective affinity for the ion in the occluded state is then greater than the intrinsic affinity of the exposed site.

What is the significance of occluded states? Present views of protein structure and dynamics hold it unlikely that rotation of the transport pocket is the mechanism of translocation, but favor schemes in which a binding site is presented first to one side of the membrane and then to the other by means of minor modifications in the protein structure. When the sites are exposed to the extracellular medium ion movement must be blocked by a barrier on the intracellular side, and similarly in another conformation when the sites are exposed to the intracellular medium, movement must be blocked by a different barrier on the extracellular side. One possibility is that the position of both barriers is determined by a single conformational change of the protein with one opening and the other closing in the same event; in this case, there would be no occluded states. If on the other hand, the position of the two barriers reflects two distinct and partially independent conformational changes, then occluded states will consist of states in which both barriers are closed. Note that the rules of allowed protein conformational changes must specify that both barriers can not open at once, or else the transporter will leak ions from one side of the membrane to the other (Post *et al.*, 1969, Figure 22).

OCCLUSION IN THE TRANSPORT OF K.

Evidence for occlusion of K and for the occluded state as an intermediate in transport.

Early in the characterization of Na,K-pump fluxes it became clear that ion interactions with the pump were important during the translocation process, since the *rate* of translocation of various K congeners was found to depend on the species of cation but to be unrelated to the *affinity* for the ions in binding at the extracellular side of the pump (Sachs, 1967). We now know that the ionic species dependence of the transport rate seen in those experiments reflected the interaction of occluded ions with the protein. Post *et al.* (1969) and Glynn and Hoffman (1971) suggested that during the translocation process an intermediate might be identified in which the ions were buried within the membrane, and soon thereafter Post *et al.* (1972) presented very strong evidence that such a state occurs during K translocation. In examining the requirements for phosphorylation from [γ-^{32}P]ATP, Post *et al.* showed that an exposure to K or Rb prevented subsequent phosphorylation of Na,K-ATPase: the Na,K-ATPase apparently "remembered" the prior exposure to the cation, since the effect was still manifested after dilution of the medium. A number of cations could be distinguished, and it seemed unlikely that this could be accomplished by a multitude of protein conformations that might remain after dissociation of the various cations; Post *et al.* (1972) therefore proposed that the cations did not dissociate, but remained bound to the protein in an occluded state.

The same set of experiments provided the explanation for the requirement of the Na/K pump for ATP at a much higher concentration than that necessary for formation of the phosphorylated intermediate. Post *et al.* (1972) found that high concentrations of ATP relieved the block of phosphorylation caused by prior exposure to Rb, and they hypothesized that in the normal operation of the pump, ATP binding is necessary in order to promote the release of occluded K to the intracellular medium, accompanying the transition from the E_2 form to the E_1 form of the enzyme.

The behavior of the Na,K-pump in its ^{86}Rb-Rb exchange mode has provided a strong parallel to the behavior of the ^{86}Rb-occluded state of Na,K-ATPase. The requirement for ATP at a low affinity site to promote deocclusion of K or Rb is also seen in ^{86}Rb-Rb exchange, a partial reaction in

which ATP hydrolysis does not occur. ADP can promote ^{86}Rb-Rb exchange almost as well as ATP (Kaplan and Kenney, 1982), and indeed ADP has been shown to be capable of promoting the release of occluded ^{86}Rb (Glynn and Richards, 1982) about half as well as ATP (Forbush, 1987b). Inorganic phosphate (with magnesium) is the second ligand required for optimal ^{86}Rb-Rb exchange, and its action can be understood as bringing about a reversal of the steps in which Rb is bound to the E-P intermediate from the extracellular medium. Significantly, inorganic phosphate is effective in promoting rapid ^{86}Rb release from the ^{86}Rb-occluded form of Na,K-ATPase (Glynn and Richards, 1982; Forbush, 1987c). Finally, from the kinetic behavior of ^{86}Rb-Rb exchange it has been concluded that it must be possible for both ATP and P_i to bind to the pump at the same time (Sachs, 1981; Eisner and Richards, 1983; Karlish *et al.*, 1982); similarly nucleotides and P_i are found to bind at the same time in bringing about ^{86}Rb deocclusion (Forbush, 1987c).

While for the most part the sidedness of ^{86}Rb occlusion has been inferred from correlative ion flux studies in intact systems, two recent studies have supported the proposed sided relationships of the occluded state. In this volume Kenney and Kaplan report experiments with red cell ghosts in which they replaced K with Li, which is transported but is not retained for long in the occluded state (Post *et al.*, 1972). They find that when Li is transported in the inward direction, ATP is not required at a low affinity site in order to obtain the maximal rate; conversely, when Li is transported outward, P_i is not required. In a different approach, we have directly studied the release of occluded ^{86}Rb from Na,K-ATPase in tight right-side-out vesicles. We loaded the vesicles with inorganic phosphate but no magnesium, and formed the occluded state with 80 μM ^{86}Rb; the extravesicular medium contained ATP in order to prevent possible interference from broken membranes. When phosphorylation is initiated by the A23187-promoted introduction of magnesium into vesicles which already contain inorganic phosphate, ^{86}Rb is found to be rapidly released to the *outside* of the vesicles (Forbush, unpublished data); this is consistent with the proposed role of ^{86}Rb occlusion in transport and the reversal of transport upon phosphorylation from inorganic phosphate.

Deocclusion as a rate limiting step in the pump cycle.

The work of Post *et al.* (1972) demonstrated that high concentrations of ATP were necessary to speed the break down of the K-occluded form of Na,K-ATPase, but these experiments did not indicate how fast the maximal rate of deocclusion might be, and therefore whether or not deocclusion is rate limiting under optimal pumping conditions. Karlish and Yates (1978) showed that the form of the enzyme that is stabilized by K, $E_2(K)$, has slightly higher intrinsic fluorescence than E_1 forms; using a stop-flow apparatus they were able to measure the rate of the ATP-stimulated $E_2(K) \rightarrow E_1$ conversion as ~$18s^{-1}$ at 100 μM ATP, and using an estimated value of the K_d for ATP (450 μM), extrapolated the maximal rate of deocclusion to 54 s^{-1}. Compared to a turnover rate of ~30 s^{-1} at 24°C, this was clearly fast enough for the deocclusion reaction to be in the pump cycle, but slow enough that it would constitute one of the principal rate limitations.

When it became possible to directly measure the occluded ions, Glynn and Richards (1982) showed that ATP and ADP promoted the rapid release of ^{86}Rb from Na,K-ATPase. Using the rapid filtration apparatus, we have been able to directly determine the rate of breakdown of $E(^{42}K)$ to be ~$45s^{-1}$ at high concentrations of ATP (Forbush, 1987b) compared to an overall turnover rate of about $20s^{-1}$ at 20°C. Thus, in agreement with the finding of Karlish and Yates (1978), the rate of deocclusion accounts for approximately half of the overall rate limitation in Na,K-ATPase activity under normal conditions.

^{86}Rb deocclusion is not rate limiting under all conditions, as is seen by examining the system at different pH values (Forbush, 1987b). The pH dependence of the overall ATPase rate is bell-shaped, with an optimum between pH 7 and 8 (Skou, 1957). Below pH 7.5 the decrease in rate with pH can be accounted for by deocclusion as the rate limiting step, since ^{86}Rb release in the presence of ATP falls as the pH is lowered. However above pH 8 the rate of deocclusion increases with pH and plateaus, in a range in which the ATPase activity declines. Thus at high pH, ^{86}Rb deocclusion is no longer a significant rate limiting step in the Na,K-pump cycle, and we have proposed that Na transport steps must be rate limiting. Consistent with this finding, the steady state level of the ^{86}Rb-occluded form is maximal at low pH and lower at high pH. The difference between Rb-stimulated and K-stimulated ATPase

activity also reflects the switch in rate limiting steps: at lower pH values Na,Rb-ATPase activity is less than half of Na,K-ATPase activity, mirroring a 2- to 3-fold lower rate of ^{86}Rb deocclusion compared to ^{42}K deocclusion; however at high pH, where deocclusion is not rate limiting, the ATPase activities in K and Rb are about the same (Forbush, 1987b).

Two sites for occlusion of K.

The Na,K-pump is known to transport three Na ions and two K ions in one cycle in which one ATP molecule is consumed; it is not surprising therefore that two ^{86}Rb ions can be occluded in the transport sites of one functional Na,K-ATPase unit (Glynn *et al.*, 1985b; Forbush, 1983, 1987b). ^{86}Rb ions at the two sites can be distinguished by the characteristics of their release from the phosphorylated enzyme: the release of one of the two ions, from what we term the "f" site, is rapid in the presence of either Na or K, whereas the release of the other ion from the "s" site is slowed greatly by the presence of K or Rb in the medium. This has been seen as the release of only about half of the total ^{86}Rb into a K-containing medium in 0.9s in the ion-exchange apparatus of Glynn and coworkers (Glynn *et al.*, 1985a,b), and in a markedly biphasic time course of release in the rapid filtration apparatus (Forbush, 1985, 1987c). The affinity for K in blocking release from the "s" site is high (~0.1 mM), and the specificity is that of the extracellular K transport site. It was therefore proposed in these studies that ions can be released from the "s" site only when the "f" site is unoccupied and that after release of ^{86}Rb from the "f" site, the "f" site can immediately become reoccupied by K ions from the medium. This model has been supported by further experiments which show that the ion that blocks release from the "s" site can itself become occluded (Forbush, 1987c), and that with right-side-out vesicles, it is indeed extravesicular K or Rb that blocks half of the P_i-promoted release of occluded ^{86}Rb (Forbush, unpublished results).

When deocclusion occurs in the forward transport mode stimulated by ATP, no difference is observed in the rates of release of ^{86}Rb from the two sites, even when the sensitivity of the test is enhanced by placing radiolabeled Rb in one or the other of the two sites by sequential preincubation (Forbush, 1987c). This is unfortunate,

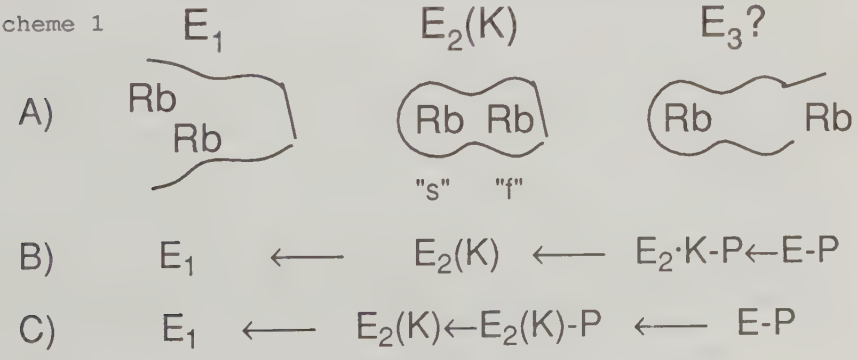
because we can not answer the question as to whether transport of the two Rb ions proceeds as a "first-in-first-out" ordered process as we might expect. The finding is easily rationalized by noting that it is probably the $E_2 \rightarrow E_1$ conformational change that is rate limiting in the ATP-stimulated deocclusion process, and that the dissociation of both ions is probably much faster than the conformational change; once the conformational change takes place, the ions are both released immediately.

The behavior of the Na,K-ATPase is affected by the nature of the ions in the K-occlusion site, as shown by the stability of the occluded state with various K congeners (Rb,Cs > K,Tl in stability; Post *et al.*, 1972). It is interesting that ions in the "f" site are more influential in this interaction than ions in the "s" site -- when one ^{86}Rb ion and one unlabeled cation are placed in the occlusion sites by sequential incubations, the differential effect (K > Cs > Tl > Rb in stimulation) on the ATP-stimulated rate of ^{86}Rb release is greatest when the unlabeled cation is in the "f" site and the ^{86}Rb ion is in the "s" site (Forbush, 1987c).

What are the ^{86}Rb-occluded states?

The concept of occluded states as intermediates in ion transport raises an important point: there must be protein barriers to ion release on both sides of the membrane, and in order for access to be granted to the ions, the barriers must be moved out of the way; in other words, conformational changes must occur, however small. As summarized above (and see also Glynn *et al.*, 1987), the $E_2 \rightarrow E_1$ conformational change accompanies exposure of the K sites to the extracellular medium. However a conform-

Scheme 1



ational change involved in exposure of the K sites to the extracellular medium ($E_2 \rightarrow E_3$? in Scheme 1A) has not been discovered to date. A "flickering gate" model is discussed below in which the transport sites are exposed to the extracellular medium only very briefly, ie. the lifetime of the hypothetical "E_3" state is very short.

In many models of K transport, the change from extracellularly facing sites to occluded sites is presumed to accompany dephosphorylation (Scheme 1B, Post *et al.*, 1972; Karlish *et al.*, 1978; Glynn *et al.*, 1984; Glynn, this volume). Our data regarding the interaction of phosphate and arsenate with the occluded state are incompatible with this formulation, but suggest instead that the K-occluded intermediate may be phosphorylated (Scheme 1C; Forbush, 1987c, 1988b). The observations include the finding that when the enzyme is phosphorylated from P_i the ^{86}Rb-occluded state can be formed at equilibrium, and that the occluded form can be phosphorylated from $^{32}P_i$; furthermore the kinetics of ^{86}Rb release reveal a reaction step that appears to reflect phosphorylation, asynchronous with ^{86}Rb release. We can account for the effects of phosphorylation or arsenylation in a model in which formation of the phosphorylated state increases the frequency with which the transport sites become exposed to the extracellular medium. An important consequence of the difference between models in Scheme 1B and 1C is that as compared to the conventional sequence K-binding→dephosphorylation→K-occlusion→deocclusion, we propose that the normal sequence in K transport is K-binding→occlusion→dephosphorylation→deocclusion (omitting ATP binding, which may occur at any step, but is necessary for the last step).

A model of the interaction of K-occlusion sites with the extracellular medium.

The nature of Rb interaction at two occlusion sites on the phosphorylated form of Na,K-ATPase is quite unusual, and can not be explained by a conventional model of a slow conformational change and rapid ion dissociation (which does appear to fit the data regarding ATP-stimulated release to the intracellular medium). We have proposed a "flickering gate" model in which access of the ion binding sites to the medium occurs infrequently and briefly, long enough for an ion to leave the "f" site, but not long enough to allow the ion to leave the "s" site. As with any modeling, it is not possible to prove that this is the

correct model, only that certain other simple models do not account for the data. The conclusions we have reached are outlined below, along with a brief summary of the relevant data, and some discussion.

1) The process of ion release from two sites is ordered. The clearest demonstration of this is that when Na,K-ATPase is preincubated in such a way that ^{86}Rb is in the "s" site, and unlabeled Rb is in the "f" site, the release of ^{86}Rb ion follows a lag during which Rb is released from the "f" site (Forbush, 1987b, Fig. 7).

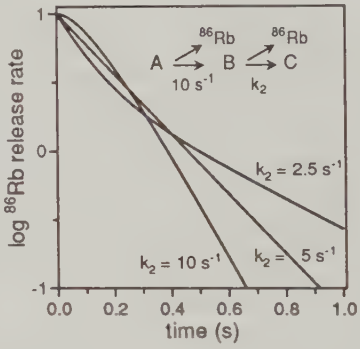
2) The process can not be modeled by a slow conformational change followed by rapid dissociation of two ions from the binding sites. If the rate limiting step is a conformational change and both ions dissociate rapidly, then even if release is ordered, both ions will appear in the medium together following the conformational change. For the same reason, the block of release from the "s" site would not be observed except at very high K concentrations (with an apparent $K_{0.5}$ for K being greater than the K_d for occlusion by a factor approximately equal to the ratio of the rate constants for dissociation and the conformational change (see Discussion in Forbush, 1988c). *Thus, at least one slow step must occur after Rb dissociation from the "f" site and before Rb dissociation from the "s" site.*

3) The same step is rate determining in the release of ions from both the "f" site and the "s" site. Suppose the converse to be true, that two different steps limit the rate of release from the two sites; this would be the case if release of the first ion were limited by a slow conformational change (with rate constant k_1), and release of the second ion were limited by a step involving a "jump" from the "s" site to the "f" site (k_2). For the reasons outlined in (2), k_2 can not be much greater than k_1. If k_2 is appreciably less than k_1, the rate of dissociation of ^{86}Rb would be markedly biphasic (even in the absence of K), reflecting the two rate constants. However the data show that all of the ^{86}Rb release in the presence of choline occurs in essentially a single phase (Forbush, 1987c); furthermore, when the rate of k_1 is changed over a very wide range of conditions (vanadate inhibition, pH 6-9, 0°-35°C) the shape of the ^{86}Rb release curve remains essentially monophasic (Forbush, 1987c; and unpublished observations). This contradicts the proposition that two independent rate constants are involved and argues instead that a single step limits the rate of release of each of

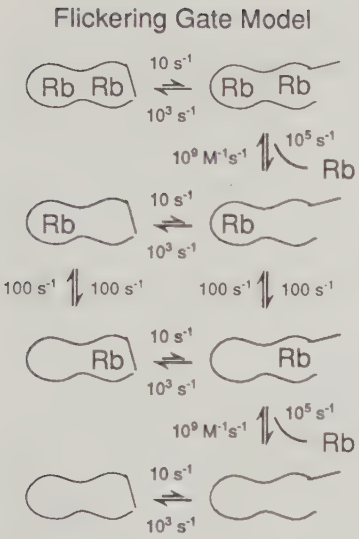
the two ions: it is simplest to propose that this step involves access of the ions in the pocket to the extracellular medium.

As illustrated in figure 1, if ^{86}Rb is released in two sequential steps, then the overall rate of release is described by a single exponential only when $k_2 = 0.5 * k_1$. At first it may seem that this should occur instead for $k_2 = k_1$, but it can be appreciated that then as the first ^{86}Rb ion was released there would be no change in the overall rate of release since the second ion would be released at the same rate (shown in figure 1). If the same step determines both k_1 and k_2, how can the apparent rate constants be different by a factor of two? A reasonable explanation is that when only one ion is left in the pocket it may have an equal chance of being in either the "f" site or the "s" site; then if the rate determining factor is exposure of the "f" site to the medium the apparent rate constant for the ion leaving the pocket would be half that of when the pocket is fully occupied, and overall ^{86}Rb release would exhibit a monoexponential decay.

Figure 1.



We have considered two types of mechanisms whereby a single rate limiting step could regulate the sequential release of each of the ions from the transport pocket (Forbush, 1987c). In the "leaky pocket" model, ions are able to leak out of the pocket slowly, as they might if they had to pass a fixed opening that limited their access; in this model the "f" site is never freely exposed to the medium. In a second model (scheme 2), a "flickering gate" controls the rate of access to the medium: it opens infrequently and for only a short time, but while it is open the ion in the "f" site can rapidly exchange with the medium. Thus in the leaky pocket model the overall rate of release is given by the rate of leak of the ion from the pocket, whereas in the flickering gate model the rate of release is determined by the frequency of opening of the gate. Note that for simplicity it is assumed that the mechanism regulating access to the sites is not affected by the occupancy of the sites.



Scheme 2.

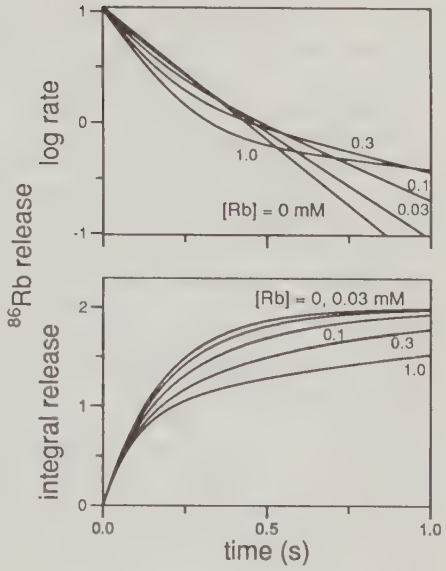


Figure 2.

Figure 2 presents predictions of the flickering gate model obtained by numerical integration of the set of differential equations expressing the model in Scheme 2 (additional states are included to for occupancy by unlabeled Rb as is a spontaneous background release of 0.3 s^{-1}). The predictions shown here illustrate that the model can account for the essential features of ^{86}Rb release in the absence and presence of various concentrations of extracellular Rb; similar solutions for the leaky pocket model (not illustrated) have demonstrated that this model as well can fit the data for ^{86}Rb release. On the other hand, the two models make different predictions with regard to formation of the occluded state upon incubation of phosphorylated Na,K-ATPase with ^{86}Rb. In the leaky pocket model, access is through a restricted opening, and the rate of entry of ions into the pocket is proportional to the concentration of ^{86}Rb in the medium, even above the concentration range in which the sites are saturated at equilibrium. In the flickering gate model, the rate of ^{86}Rb entry is determined by the frequency with which the gate opens in the same way as is ^{86}Rb release; the rate is therefore independent of ^{86}Rb concentration. These differences are illustrated by the numerical solutions in figure 3A.

4) The rate of formation of the occluded state is limited by the rate of the slow step regulating exposure of the occlusion sites and does not depend on the ^{86}Rb concentration in the medium. We have determined the rate of formation of the ^{86}Rb-occluded state of Na,K-ATPase at 0°C by manual filtration (Forbush, 1987b), because "on" rates can not be determined in our rapid filtration apparatus. The combined data from 2 identical experiments (averages ± S.E.M., n=6) are shown in Figure 3B. The rate of ^{86}Rb occlusion is seen to be essentially constant over a 15-fold range of [^{86}Rb]. This is inconsistent with the predictions of the leaky pocket model, but is in agreement with those of the flickering gate model.

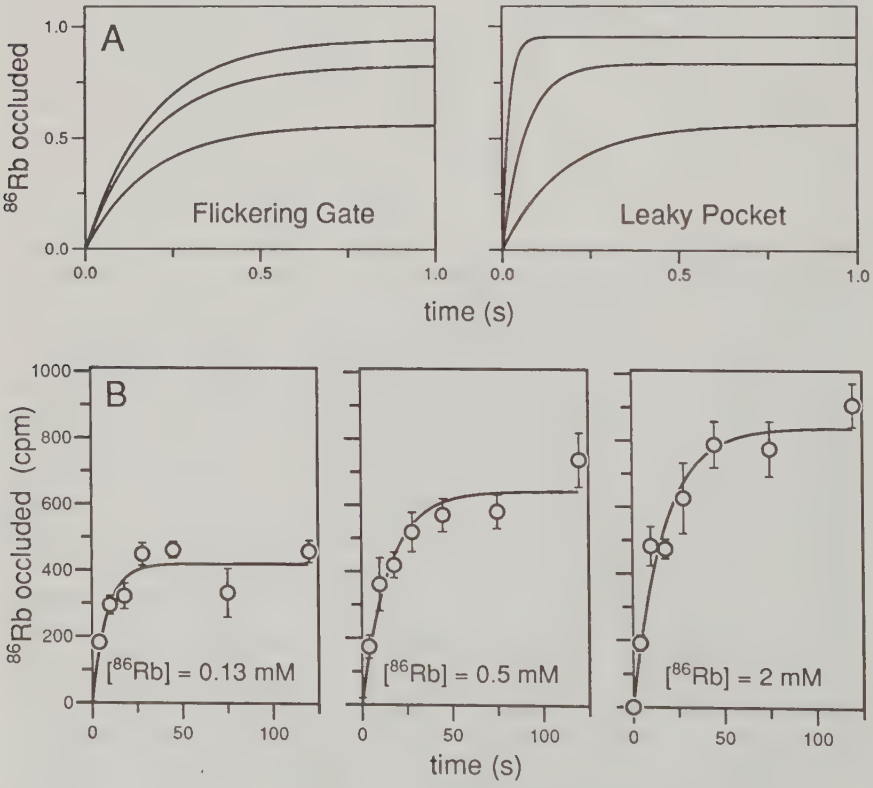


Figure 3. Formation of the ^{86}Rb-occluded intermediate. A. Predictions of the flickering gate model (Scheme 2), and the leaky pocket model. B. Observed rate of formation at 0°C (see text).

In summary, the interaction of the K-occlusion sites of Na,K-ATPase with the extracellular medium can be explained if a single step limits the rate of ion release from two occlusion sites. Furthermore, it is proposed that this single step regulates access of the "f" site to the medium, and that the mechanism of access involves the brief opening of a barrier that transiently exposes the site to the medium.

The effect of extracellular Na

Extracellular Na acts at a high affinity site to modify many aspects of pump activity (for references and discussion see Sachs, 1987). We have found that Na prevents K or Rb from blocking release of ^{86}Rb (Forbush, 1987b; K_{Na} = 2 mM). But Na does more than stop K from binding to the "f" site -- it actually promotes immediate release of a lone ^{86}Rb ion from the "s" site. The most dramatic manifestation of this occurs after a preincubation of the ^{86}Rb-occluded form with quaternary amine and MgP_i (see below) which causes the release of the ^{86}Rb ion from the "f" site and leaves the "f" site empty; the release of the remaining ^{86}Rb then occurs at about 5 s^{-1} in choline, but at >100 s^{-1} in Na (Forbush, 1988c, and unpublished results; K_{Na} ~ 1 mM). The same effect is reflected in the fact that the rate of release of ^{86}Rb in $NaMgP_i$ is twice the rate in $cholineMgP_i$; this is consistent with Na causing immediate release of the ^{86}Rb ion from the "s" site after the "f" site is unoccupied (Forbush, 1987c, and unpublished results). An important point is that Na appears to have no effect on occluded ^{86}Rb when *both* occlusion sites are occupied.

Models of ordered release of ^{86}Rb from extracellular sites predict that during ^{86}Rb-Rb exchange, only one site is effectively used in transport, the "s" site always remaining occupied by Rb from the intracellular medium (Forbush, 1985); since Na can cause the release of the ion from the "s" site, it is possible that low concentrations of Na would *stimulate* the rate of ^{86}Rb-Rb exchange. At higher concentrations, Na should be inhibitory because it prevents reformation of the fully occluded state. Recent experiments of Kenney and Kaplan (personal communication) have shown just that: in resealed red cell ghosts, in the presence of 250 μM Rb_{ext}, Na_{ext} stimulated ^{86}Rb uptake at concentrations below 5 mM and inhibited at higher concentrations, consistent with the prediction.

OCCLUSION IN THE TRANSPORT OF Na.

It seems fairly certain that Na-occluded states take part in the Na transport pathway. However, unlike the ease with which the K-occluded intermediate can be studied, it has not been possible to make a direct observation of the Na-occluded form in the uninhibited Na,K-pump. The presumed difficulty is that once the Na transport process is set in motion, the Na transport steps proceed rapidly to completion with release of extracellular Na. By blocking the sequence of reactions at the E_1-P$\rightarrow E_2$-P step using chymotrypsin or NEM, Glynn *et al.* (1984) were able to show that ^{22}Na could become occluded on Na,K-ATPase, and that the occluded ^{22}Na ions were released upon reversal of phosphorylation by ADP addition; in the rapid filtration apparatus the release is seen to take place rapidly (~60s^{-1}; Forbush, 1985). These are characteristics expected of an E_1(Na)-P intermediate, and it is argued (Glynn *et al.* (1984)) that this occluded intermediate takes part in the normal cycle. The oligomycin-inhibited enzyme can occlude Na without being phosphorylated (Esmann and Skou, 1985). While these investigations illustrate that Na,K-ATPase can occlude Na, there remains the possibility that in the uninhibited pump, closing of the intracellular Na-occlusion barrier and opening of the extracellular barrier occur as concerted events -- in this case an occluded state would not be formed.

In a single turnover of the Na,K-pump, ^{22}Na is released with a burst that has a decay rate of 25-40 s^{-1} at 20°C (Forbush, 1984a, 1985), accompanied by a burst of electrical current with essentially the same time course (Borlinghaus *et al.*, 1987; see also Fendler *et al.*, 1987). Although there is ambiguity in the assignment of the observed rate constant to a particular step in the reaction cycle, a possibility is that this is the rate constant of release of ^{22}Na. The rate is considerably lower than the rate of E_1-P$\rightarrow E_2$-P estimated in Na,K-ATPase from electroplax (>100s^{-1}; Froehlich *et al.*, 1976) and brain (>60 s^{-1}; Mårdh, 1975). Taken at face value the discrepancy suggests the possiblility that Na remains bound to the protein for some time after the E_1-P$\rightarrow E_2$-P conformational change, and thus that an E_2(Na)-P occluded form exists. Clearly, an understanding of the role of occluded ions in Na transport awaits a better resolution of the time course of phosphorylation, of conformational changes and of Na binding and release.

OTHER IONS IN THE OCCLUSION SITES

Ca in K sites.

Distinct from the role of Mg or Ca at divalent cation sites, Ca has been found to mimic K in formation of a stable state that on subsequent phosphorylation from $^{32}P_i$ is able to dephosphorylate rapidly -- ie. $E_2(Ca)$-P is formed instead of $E_2(K)$-P (Vasallo and Post, 1986). We have found that under these conditions one ^{45}Ca ion is occluded in place of the two ^{86}Rb ions and that the ATP-stimulated ^{45}Ca release is similar to the ATP-stimulated ^{86}Rb release (Forbush, 1986, 1988a). However phosphorylation with P_i, which increases the rate of release of K from the occluded state to the extracellular medium, actually stabilizes the Ca-occluded form, suggesting that a barrier in the transport pocket prevents movement of the Ca ion out to the extracellular medium (Vasallo and Post, 1986, Forbush, 1986, 1988a). One possibility is that there is a temporal rather than structural restriction (the ionic radius of Ca being smaller than that of K): if Ca ions do not dissociate rapidly enough in the brief period when the "flickering gate" is open, then they will not be released; this might be so if the single Ca ion were located at the "s" site.

When ^{45}Ca is occluded, a K or Rb ion can also become occluded, as deduced from the behavior of the Ca ion (Forbush, 1988a). Since the formation of the mixed occupancy state occurs in the presence of MgP_i, it is plausable that K enters the "f" site. The apparent affinity for K (K_{app} = 10-50 mM) is very low, indicating that if the site is indeed a normal K transport site, there is unusual steric (or electrostatic) destabilization due to the presence of Ca.

Amines in K sites.

Many organic cations act like extracellular K in blocking the release of ^{86}Rb from the "s" site on Na,K-ATPase; of a large number of compounds tested, tetrapropylamine and various benzylamines are the most effective, with affinities in the range of 2-10 mM (Forbush, 1988c). The amine molecule is presumed to occupy the "f" site, but it does not become occluded, perhaps due to steric restrictions on the gate mechanism. As long as the organic cation continues to bind to the Na,K-ATPase, the enzyme is also unable to release ^{86}Rb from the "s" site

in the presence of ATP (as with $^{45}Ca/K$ co-occlusion, above), suggesting that the $E_2 \rightarrow E_1$ conformational change is not allowed. These actions appear to be sufficient to account for the inhibition of Na,K-pump activity by tetrapropylamine described by Kropp and Sachs (1977).

The greatest potential importance of organic cation interactions is the possibility of using a suitably derivatized molecule for photolabeling the transport sites. In this regard, the low affinity of the mono-functional amines at the "f" site and the finding that the amines do not become occluded are discouraging because of the likelihood that photoaffinity derivatives would be non-specifically incorporated. On the other hand, ethylenediamine inhibits Na,K-ATPase with high affinity (Schuurmans Steckhoven *et al.*, this volume) and competes with ^{86}Rb in formation of the occluded state (K_d ~30 μM; Forbush, 1988c), suggesting that appropriate diamine derivatives may be important probes for localizing the transport pocket within the protein structure.

Na in K sites.

Inhibition by ouabain effectively "locks" the access gate to the K occlusion site, and it prevents the $E_2 \rightarrow E_1$ conformational change: we have found that ^{86}Rb release from the ouabain-inhibited enzyme is extremely slow, even at 37°C (~0.0004 s^{-1}; Forbush, 1983). The inhibited complex does respond to the presence of various ligands, including Na, K, Mg, and ATP or ADP, as evidenced by changes in the rate of release of ^{86}Rb and [^{3}H]ouabain. In similar experiments, if ouabain inhibition takes place in the presence of ^{22}Na, MgATP, and no K, we find that two ^{22}Na ions become occluded per functional unit of Na,K-ATPase (K_d ~0.7 mM; Forbush, 1987a; and unpublished observations). Since ^{22}Na occlusion takes place even when the ouabain concentration is too low to be theoretically able to "catch" the Na transport half cycle, and since the presence of K prevents ^{22}Na occlusion, it is clear that the ^{22}Na ions which become occluded must be bound to E_2-P, presumably from extracellular sites. The surprising aspect of the ^{22}Na•[^{3}H]ouabain•Na,K-ATPase complex is that the release of ^{22}Na has characteristics very similar to release of ^{86}Rb from the corresponding complex; for instance release of either cation is stimulated by ATP but not by Mg+ATP. These experiments raise, but do not answer, the following questions: a) under the conditions described, is

extracellular Na occluded at K sites, b) do Na sites and K sites have identical behavior in the ouabain-inhibited enzyme, or c) are K sites and Na sites the same, as hypothesized in the following section?

Is there but one set of transport sites alternating between Na transport and K transport in two halves of the cycle?

Simplicity of mechanical design argues strongly in favor of a single set of transport sites for Na and K, and this physical arrangement would be consistent with the "ping-pong" nature of the Post-Albers model. There is no direct evidence to support the single transport pocket hypothesis, but there is also a lack of evidence to the contrary: simultaneous effects of Na and K have been reported, but it has not been demonstrated that simultaneously bound Na and K ions are bound at transport sites.

If a single set of transport sites is accepted as part of a working hypothesis, an interesting set of questions is raised. Do the same barriers control exposure of both Na and K on both sides of the membrane? If so, and if K sites are occluded in the E_2 conformation and exposed to the medium in the E_1 conformation, then why is $E_1(Na)$-P rather than $E_2(Na)$-P an occluded intermediate? One possibility that has been considered is that the gross conformational change termed "E_1-E_2" may not be synonymous with opening and closing of the barrier (Jørgensen, 1985; Glynn *et al.*, 1987). How do Na and K ions get past one another at the extracellular "flickering gate", which in the absence of Na appears to demand single-filing of two K ions? Is the extracellular high affinity Na site one of the transport sites? What determines the specificity of the transport pocket if there is only one pocket, and what is the significance of Na ions in a K-like occluded state?

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OVERVIEW:

THE PHOSPHOINTERMEDIATES OF Na,K-ATPase

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INTRODUCTION

The hydrolysis of ATP by all the known membrane-located cation transport ATPases proceeds via a series of reactions where the γ-phosphate of MgATP is first transferred to an acid stable, anhydride bond between phosphate and the carboxyl group of an aspartic acid residue on the ATPase. The resulting phosphoenzyme is denoted EP. ADP is released as the first product from the phosphoenzyme and the -CO-O-P is then hydrolysed to yield the second product, inorganic phosphate, P_i. The similarity in reaction pattern among the transport ATPases is underlined by the observation of a considerable homology in the aminoacid sequences of the three important peptide domains involved in ATP-binding and phosphorylation (Nørby, 1987). The formation, interconversion and dephosphorylation of the phosphoenzymes seems to be closely connected with transmembrane ion transport and a characterization of these intermediates may therefore lead to a better understanding of the transport process.

For Na,K-ATPase, phosphoenzymes were first demonstrated about 25 years ago. A brief account of the early work is given by Klodos et al. (1981). Over the years the concept has developed that Na^+ transport is connected with the formation and conformational change of EP, whereas K^+ transport is initiated with the binding of K^+ to EP with subsequent dephosphorylation. This sequence is an integral part of the Albers-Post model for Na,K-ATPase and Na,K-transport (Glynn, 1985; this volume). The phosphoenzymes were believed to exist in two conformations: E_1P, which was formed first and was reactive with ADP - called ADP-sensitive EP - and E_2P, which was dephosphorylated rapidly by reaction with K^+ - called K-sensitive EP. Recently, however, it has become necessary to take a more differentiated view on the role and properties of the phosphointermediates

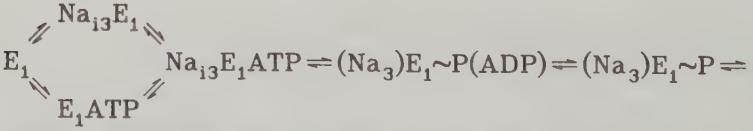
(Glynn, this volume). The present chapter will present a overview of the state of the art with an emphasis on the new observations, interpretations and theories.

PHOSPHORYLATION BY ATP AND OCCLUSION OF Na^+

The dephosphoenzyme can, as the phosphoenzymes, exist in at least two distinct conformations E_1 and E_2 (Glynn, 1985; this volume; Jørgensen, this volume). The E_1-form has an affinity for ATP in the order of 10^7 M^{-1} and in the presence of Na^+ it is in this form that the enzyme is phosphorylated by ATP (references in Nørby, 1983; Glynn, 1985). Indeed, since the rate constant for the transition from E_2 to E_1 is much lower than k(apparent) for phosphorylation of E_1, formation of phosphoenzyme can be used to monitor the kinetics of the former process (Klodos and Ottolenghi, 1985).

The preincubation experiments by Post et al. (1969), Mårdh and Post (1977) and Klodos and Nørby (1987) show that it is ATP bound to the high affinity site that phosphorylates the enzyme. It is also clear from the two latter works that preincubation with Na^+ (or $Tris^+$, Klodos and Nørby, 1987) besides ATP, further increases the "phosphorylation rate constant" observed when the missing ligands (Mg^{2+} + Na^+) are added. ATP and Na^+ add in random order to the enzyme, at least when it is brought into the E_1-form e.g. by $Tris^+$ (Jensen et al., 1984) and Na^+ acts at cytoplasmic high affinity sites (Blostein, 1979; see Stein, 1986, p 542). During the phosphorylation process the first acid-stable EP formed is the ADP-sensitive E_1~P (Post et al., 1969, 1975; Fukushima and Nakao, 1981; 1983). These findings can be summarized in the following scheme:

Scheme I



Subscript i stands for "intracellular" and a parenthesis around the ion indicates that the ion is presumed to be sequestered - occluded - in the enzyme. The role of Mg^{2+} is not shown. Beyond the Mg^{2+} complexed with ATP there is probably one Mg^{2+} bound to a site on the enzyme (refs. in Forbush, 1987, p. 11126).

It is important already at this stage to discuss how many Na^+ ions are occluded. Modelling of the entire sequence requires detailed consideration of this number, since these are the ions that at a later stage must be deoccluded to extracellular aspects of the system. The number of Na^+ ions involved is here set to 3 and this is based on a number of different observations, although the relationship between phosphorylation rate and Na^+ concentration is not very well characterized (Mårdh and Post, 1977). Direct binding studies suggest binding of 3 Na^+ per ouabain site (Matsui and Homareda, 1982), and in the presence, but not in the absence, of oligomycin, Esmann and Skou (1985) found at least 3 Na^+ occluded in the E_1 conformation. The studies of Glynn et al. (1984) with NEM- or chymotrypsin-treated enzyme - measures that lead to accumulation of E_1~P - and of Vilsen et al. (1987; this volume), who phosphorylated the enzyme with CrATP, both indicate that about 3 Na^+ are occluded in the E_1~P conformation as shown in scheme I. Bearing on the stoichiometry for Na^+ in this sequence are also the studies concerned with the different modes of Na-transport (for refs. see Nørby et al., 1983, p. 741 and 746) notably the observations of 2-3 Na^+ per ATP in uncoupled Na^+ efflux from red blood cells (Glynn and Karlish, 1976). Also in the evaluation of ATP-hydrolysis as a function of cytoplasmic Na^+ in inside-out red cell vesicles (Blostein, 1983) and in a reconstituted vesicle preparation by Cornelius and Skou (this volume) the result is that for Na-ATPase activity at least 3 Na^+ ions must be bound to internal sites.

Having accepted that Na^+ is occluded in $(Na_3)E_1$~P we may ask at which stage in the process Na^+ became occluded? In the above-mentioned experiments of Glynn et al. (1984) with NEM and chymotrypsin-treated enzyme, occluded Na^+ was not detected in the absence of Mg^{2+} or with non-phosphorylating analogues of ATP, and addition of ADP to the assay led to rapid release of occluded Na^+, corresponding to a net reaction in scheme I from the right all the way to the left (Klodos and Nørby, 1987). It is probable that Na^+ becomes occluded as soon as the γ-phosphate of ATP is transferred to the enzyme and before, rather than after, ADP leaves the substrate site. With CrATP it appears that tight, acid stable complexes EATPCr, E~P(ADP)Cr and E~PCr are formed (Pauls et al., 1980) and also here it is likely that Na^+ occlusion occurs concomitant with the hydrolysis of ATP.

Thus it may well be that it is the formation of the aspartyl-phosphate covalent bond that closes the internal gate and leads to Na-occlusion. The net direction of Na^+

movement and the catalytic process is then determined by the fact that ADP leaves the enzyme into a low ADP medium, since the equilibrium constant between EATP and E~P(ADP) is not far from unity in the ATPase reaction (Karlish et al., 1978).

Phosphorylation by ATP in the Absence of Na^+

The coupling of phosphorylation and Na^+ occlusion inferred from scheme I is not strict. Under certain conditions - 50 mM imidazole HCl, pH 7, 10-100 μM Mg^{2+} and 20 μM ATP - rabbit kidney Na,K-ATPase maintained a 70% steady-state level of EP compared to the level with Na^+ (Schuurmans Stekhoven et al., 1985). The EP formed is very sensitive to K^+ (Schuurmans Stekhoven et al., 1986a). It is not possible to say at the moment whether imidazole-H^+ becomes transiently occluded. Transport studies have not be performed.

The imidazole-H^+ activated phosphorylation has a pH-optimum < 6 and there is presently a debate whether the phosphorylation is actually promoted by H^+ or by the buffer cation (Schuurmans Stekhoven et al., 1986b; Hara and Nakao, 1985). Indeed, proton interaction with the sodium pump with a pH-optimum less than 6 has been observed as a net flux in the absence of K^+ (and Na^+ of course) by Hara and Nakao (1986) and as an H^+-activation of Rb^+ (K^+) pumping (Blostein, 1985).

THE CLASSICAL TWO-POOL MODEL FOR EP

In the traditional modelling of the Na,K-ATPase reaction the formation of ADP-sensitive E_1~P with occluded Na^+ - or in the pre-occlusion era, with Na^+ bound to internal cytoplasmic sites - was followed by a conformational change from E_1~P to E_2P, which has extracellularly located sites. Subsequently Na^+ was released from these low affinity sites and E_2P could bind K^+ whereby dephosphorylation was greatly accelerated. According to this, the phosphoenzymes were considered as a two-pool system : E_1~P and E_2P or "ADP-sensitive EP" and "K-sensitive EP":

Scheme II

$$\rightleftharpoons (Na_3)E_1\sim P \rightleftharpoons Na_{o,0-3}E_2P \longrightarrow$$

$Na_{o,0-3}E_2P$ should be thought of as a pool of rapidly interchanging E_2P's with 0-3 Na^+ bound to extracellular sites. The size of the two pools was determined from dephosphorylation experiments with added ADP. Such experiments show a rapid disappearance of part of the EP, followed by a slow, apparently monoexponential, phase and E_1~P is then EP(total) minus the extrapolated intercept of the slow phase in a semilog plot. E_2P was estimated as the intercept value of such plots or perhaps more directly from similar K-dephosphorylation experiments (Figure 1). The fact that both the ADP- and the K-dephosphorylation curves show at least two different exponential decays both at 0°C and at room temperature (e.g. Mårdh, 1975; Klodos et al., 1981; Hara and Nakao, 1981; Yoda and Yoda, 1982; Froehlich et al., 1983; Nørby et al., 1983; Hobbs et al., 1985), means that, in the two pool model, the rate constants for the interconversion E_1~P $\rightleftharpoons$ E_2P are smaller than those for ADP- or K-dephosphorylation and also small enough relative to the time-resolution to permit detection of the slow phases. The meaning of the intercepts and slopes of the dephosphorylation curves is of course model dependent. For the two-pool model the mathematical expression predicts that (100 - intercept) is smaller than or equal to E_1~P or E_2P in ADP- and K-dephosphorylation experiments, respectively (Klodos et al., 1981).

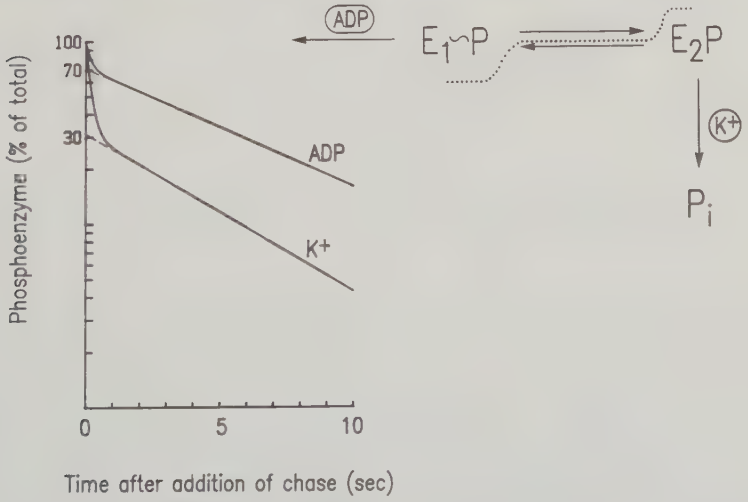


Figure 1. Hypothetical dephosphorylation curves with ADP and K^+, corresponding to a two-pool model for the phosphointermediates of Na,K-ATPase.

Already in the paper by Klodos et al. (1981) we stated, that the two-pool model could not explain certain effects of variation in Na^+ (see later). The insufficiency of the model was also readily apparent from several dephosphorylation experiments with ADP and K^+ where the sum of ADP-sensitive EP and K-sensitive EP was larger than total EP (Kuriki and Racker, 1976; Yoda and Yoda, 1982; Nørby et al., 1983; Klodos and Nørby, 1987). Although intuitively attractive explanations, based on the two-pool model, for this observation have been forwarded, it should be stressed that, when analysed mathematically, there is no way in which the two-pool model can yield (ADP-sens. + K-sens. EP) larger than 100% (Klodos et al., 1981). Figure 2 shows two detailed dephosphorylation studies that are incompatible with the two-pool model. Here ADP- and K-sensitive EP's were determined by extrapolation of the slow phases, whereas Yoda and Yoda (1982, 1986) estimate "E_1P" and "E_2P" on the basis of one time point only, i.e. after 1 sec. dephosphorylation.

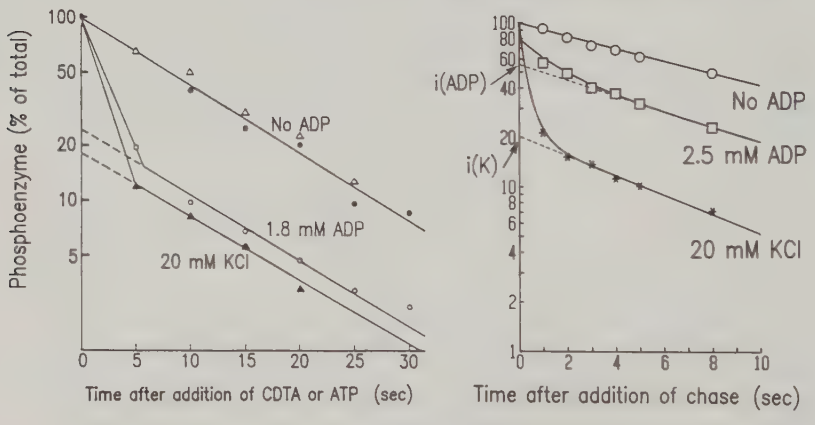


Figure 2. Dephosphorylation curves showing that "ADP-sensitive EP" + "K-sensitive EP" is larger than 100%, namely 160% (left) and 125% (right). **Left:** Na,K-ATPase from eel electroplax phosphorylated with 40 μM $AT^{32}P$, 120 mM NaCl, 5 mM $MgCl_2$ and chased with 9 mM ATP (△,▲) or 18 mM Tris-CDTA (o,●). Reprinted, with permission, from Y. Kuriki and E. Racker, Biochemistry 16: 4951-4956. Copyright 1976, American Chemical Society. **Right:** Ox-brain enzyme phosphorylated with 25 μM $AT^{32}P$, 150 mM NaCl, 1 mM $MgCl_2$ and chased with 1 mM ATP (o,*) or 2.5 mM ADP. The curve-fit is based on the three-pool model (Figure 3). Data from Nørby et al. (1983).

THE THREE-POOL MODEL FOR THE PHOSPHOINTERMEDIATES

To resolve these problems we proposed a model with more than two EP-species in succession and we showed that our results could be accurately fitted to a three-pool model which of course is the minimal alternative model to the classical two-pool concept. The model is illustrated by the upper panel in Figure 3 and this model will henceforth be used in the present chapter. Note that E_bP and E_cP are pools of EP's with 0-3 Na^+-ions bound to extracellular sites.

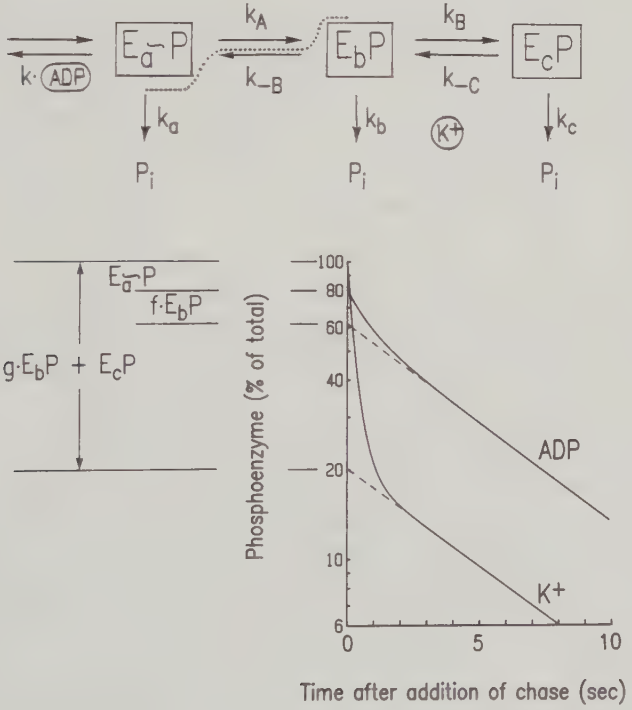


Figure 3. Three-pool model (upper panel) for the phosphointermediates of Na,K-ATPase, and principles in analysis of dephosphorylation curves (lower panel). Note that part of E_bP, namely $f \cdot E_bP$ where $f \leq 1$, is included in the first phase of "ADP-dephosphorylation". Both E_cP and E_bP are K-sensitive, but at very high Na^+-concentrations some E_bP may escape to E_a~P ($g \leq 1$). For further details see the text and Nørby et al. (1983). Reprinted with permission from Biochemistry 15, pp.4951-4956 (1976).

The necessity of the three-pool model has been substantiated in two other studies namely by Lee and Fortes (1985) who studied anthroylouabain binding (see also Fortes et al., this volume) and by Taniguchi et al. (1986).

While there seems to be a consensus in favor of the three-pool concept as a compulsory replacement of the classical models (Glynn, this volume), there are considerable deviations with respect to the way in which this model is used in evaluating dephosphorylation kinetics, Na-ATPase activity and Na-transport. The mathematical model corresponding to the three-pool model is a sum of three exponentials and estimation of the pool sizes and rate constants requires that detailed information on the system is available (Nørby et al., 1983; Nørby, 1985). Without such information it is not possible to evaluate binding affinities for Na^+ and thereby characterize the external aspects of Na-transport. We shall discuss these issues in the following sections.

Determination of the Proportion of the Three EP-Pools

It will appear from this section that the assumptions and observations regarding the reactivity of the EP-species towards ADP and K^+ are crucial for further evaluation of the proportion and properties of the phosphoenzymes in the three-pool model. First of all it is important that more than 95% of the EP formed in the presence of Na^+ is ADP- or K-sensitive (Fukushima and Post, 1978; Yoda and Yoda, 1982; Taniguchi et al., 1986; Klodos and Nørby, 1987).

Now, in order that (ADP-sensitive + K-sensitive EP) > 100%, all three EP-pools must be present in a significant amount. Furthermore, at least one of the pools must effectively be both ADP- and K-sensitive. Both Yoda's group (their terminology in parenthesis) and we consider E_bP (E^*P) and E_cP (E_2P) to be K-sensitive and $E_a{\sim}P$ (E_1P) to be ADP-sensitive.

The crucial point of difference is the reactivity of E_bP towards ADP. Yoda and Yoda (1986, p.1152) suggest that ".... $^{O}NaE^*P$ have an active ADP site.", while we, on the basis of observations discussed below, assume that E_bP cannot react directly with ADP but can be converted to $E_a{\sim}P$ with a rate that depends on the Na^+-concentration. In our model, determination of E_bP and the other two species is based on a numerical solution of three differential equations. With Yoda's assumptions, ADP-sensitive EP is $E_a{\sim}P$ + E_bP, K-sensitive EP is E_cP + E_bP, and thus E_bP is determined simply as E_bP % = (ADP-sens.EP + K-sens.EP) - 100. The two

approaches give very different results as exemplified in Table 1. The discrepancy with regard to E_bP and E_cP is so striking that it must be important to resolve the different assumptions, notably with respect to the reactivity of E_bP (E^*P) before further conclusions are drawn on the basis of one or the other procedure (e.g. see Glynn, this volume; Nørby et al., 1983; Yoda and Yoda, 1987) .

Table 1. Proportion (%) of the three EP-pools, E_a~P, E_bP and E_cP (Yoda's E_1P, E^*P and E_2P) calculated by the method of Yoda and Yoda (1986), YY, or that of Nørby et al. (1983), NKC, from the same set of experimental data (first three columns, Nørby et al., 1983).

Na^+	ADP	K	E_a~P		E_bP		E_cP	
mM	sens	sens	YY	NKC	YY	NKC	YY	NKC
20	15	88	12	12	3	46	85	42
50	27	87	13	13	14	45	73	42
150	45	81	21	20	26	40	55	40
300	67	68	32	32	35	40	33	28
600	83	38	62	50	21	35	17	15

Does E_bP (E^*P) React Directly with ADP or Is E_bP Dephosphorylated via E_a~P when ADP Is Added?

As mentioned in Introduction and shown in Scheme I and Figure 3, ADP leaves the substrate site before P_i, and the ADP-sensitive EP is formed prior to the K-sensitive. Accordingly all published models show a consecutive row of EP's with only one intermediate (pool) reacting directly with ADP. This back reaction leading to EATP (see Scheme I) is the enzymatic correlate of reversal of the pump, ADP stimulated Na:Na exchange and ADP:ATP exchange (Glynn, 1985).

Yoda's and Yoda's proposal (1986), that not only the first phosphointermediate E_a~P (E_1P) but also the second in the row, E_bP (E^*P), has an active site for ADP, is made in order to explain the observation that (ADP-sensitive EP + K-sensitive EP) > EP(total). It means that there must be two parallel back-reactions to EATP and, as a consequence hereof, also two parallel forward pathways from EATP to E_a~P and E_bP, respectively. Such an assumption is not particularly attractive, because it would reqiure that EATP "would know" to what extent it should convert to E_a~P or E_bP. The concentration of Na^+ might be a determinant in these reactions, but it would hardly be the occluded ions or those

Na^+-ions transported in ADP stimulated Na:Na exchange, since these phenomena show a fixed stoichiometry.

Yoda's and Yoda's own studies with the electroplax enzyme of transient kinetics of EP's formation at 3°C (1986, Figure 1 and 5) indicate that their assumptions and their method of calculation of pool sizes may be questioned. They find that EP(max) is reached within 0.5 sec. both with the fragmental enzyme and the proteoliposome enzyme. With the former, E_1P is also constant after 0.5 sec. but it takes more than 1.5 seconds before E^*P has fallen and E_2P risen to their steady-state levels (Figure 1). A similar pattern is observed with the proteoliposomes except that here E^*P becomes constant first, whereas E_1P and E_2P vary with time < 10 sec. It is hard to see how this is compatible with a model with three succesive, interconverting EP-pools.

There is no experimental evidence for a direct reaction of E_bP (E^*P) with ADP, and that Σsensitive EP > 100% can just as well be explained by a hypothesis where E_bP is dephosphorylated via $E_a{\sim}P$. If E_bP in ADP-dephosphorylation experiments disappears via $E_a{\sim}P$, the relative magnitude of k_{-B} (Figure 3) is decisive for the extent to which this happens. When $E_a{\sim}P$ is removed, the E_bP, E_cP system will behave as a two compartment model (Figure 1, 3) and the intercept of the slow phase will be 100 - ($E_a{\sim}P$ + f E_bP), where f is a function of the magnitude of k_{-B} relative to k_b, k_B, k_{-C} and k_c (see Figure 3 and the two-compartment analysis by Klodos et al., 1981).

There is in our opinion some experimental evidence that favors this hypothesis over the one that assumes that E_bP is directly ADP sensitive. If one takes into account that $E_a{\sim}P$ can be estimated as the intercept of K-dephosphorylation curves (Figure 3; g is close to 1 both in ours and Yoda's model), then generally at least three differential equations (or exponentials) are needed to fit the ADP-dephosphorylation curves (Nørby et al., 1983). This is easily compatible with a fast reaction of $E_a{\sim}P$ followed by a two compartment decay. It is important to note that the shape and position of these curves is independent of the ADP-concentration in the range from 1 to 10 mM (Klodos, unpublished), almost exluding the possibility that there is any directly ADP-sensitive component after $E_a{\sim}P$ has been removed. On the other hand this means that the K-sensitive EP consists of two pools and this is supported by the experiments where we, starting from the same steady-state situation, added ADP and Na^+ to dephosphorylate the EP's. We found that the slope of the slowest phase and the amount of EP that disappeared in

the faster phases increased with Na^+ (Nørby et al., 1983, Figure 4). In our analysis this means that k_{-B} is Na^+-dependent. When Na^+ is increased, k_{-B} becomes large relative to the other constants related to E_bP and E_cP, and most of the E_bP will disappear rapidly when ADP is added, i.e. ADP-sensitive EP = $E_a{\sim}P + E_bP$ (Nørby et al., 1983, Figure 6). But at low Na^+ k_{-B} is so low that f (Figure 3) is much lower than 1 and therefore the rapid phase of ADP-dephosphorylation curves is considerably less than $E_a{\sim}P + E_bP$, contrary to Yoda's and Yoda's (1986) hypothesis.

All in all, the odds in our opinion are against ascribing direct ADP-sensitivity to the E_bP (E^*P) pool, and in favor of a model where, in ADP-addition experiments, E_bP is dephosphorylated via $E_a{\sim}P$ (E_1P) with the Na-dependent rate constant k_{-B} (Figure 3).

THE KINETIC COMPETENCE OF THE PHOSPHOINTERMEDIATES

Until about 1981 "the hypothesis that hydrolysis of ATP normally proceeds through E_1P and E_2P has received support from studies of both steady-state and transient kinetics" (Glynn, 1985, who quotes 25 papers covering 1967 to 1981). But, as Glynn continues "There are also difficulties" and these difficulties we shall concentrate on in the following discussion.

When Na,K-ATPase is phosphorylated with ATP, Na^+ and Mg^{2+} in the absence of K^+, close to 100 % of the enzyme is EP in steady-state both at 0^oC (Klodos and Nørby 1979; 1987; Cantley, 1981; Nørby et al., 1983) and at higher temperatures (Jørgensen, 1974; Karlish et al., 1978). Measurement of phosphorylation sites, $E^{32}P$, can be used as a measurement of total enzyme concentration (Nørby, 1983). Under these conditions (Na-ATPase, no K^+ present) the rate coefficients obtained directly from dephosphorylation curves and the rate constants extracted from modelling of dephosphorylation kinetics, all satisfy the hypothesis that ATP hydrolysis proceeds through a number - two, three or more - consecutively connected phosphorylated intermediates (Nørby et al., 1983; Glynn, this volume).

In the presence of K^+ the situation is much more complex and let us state already at this point that there are several unsolved problems. In discussing the work on EP-levels and dephosphorylation kinetics, that might shed light on the kinetic competence of EP's in the Na,K-ATPase reaction and Na,K-transport, it is necessary to consider two principally different types of studies:

a) The enzyme is phosphorylated in the presence of Na^+ (but no K^+) and interconversion and dephosphorylation of acid-stable phosphointermediates is measured in the absence and presence of ADP or K^+ (e.g. experiments illustrated by Figure 2)
b) Phosphorylation is performed in the presence of both Na^+ and K^+ and the properties of EP's characterized by the same procedures as in a).

Hitherto we have only been concerned with phosphoenzymes produced in the presence of Na^+ alone (no K^+) and according to the widely accepted reaction scheme for Na,K-ATPase these are the phosphointermediates both in the Na-ATPase and Na,K-ATPase reaction. However, there are not only theoretical reasons (Skou, 1971; Klodos et al., 1981; Plesner et al., 1981) but also experimental evidence (Nørby et al., 1983; Glynn, 1985 and this volume) to suggest that the properties of the phosphointermediates might depend to some degree on the ions present during their formation. We shall in the following discuss the two types of studies, a) and b), separately.

Phosphoenzymes Formed in the Presence of Na^+ Alone (no K^+)

In the absence of K^+ (see Nørby et al., 1983) these phosphoenzymes satisfy the criteria for intermediates in the Na-ATPase reaction. The point to discuss is then whether they also turn over fast enough under conditions of K-stimulation. The experimental evidence, model independent, is that the K-sensitive EP's are dephosphorylated fast enough by K^+ for them to be intermediates in the Na,K-ATPase reaction cycle.

For $E_a{\sim}P$ (ADP-sensitive) also to be kinetically competent it is required that its rate of conversion to E_bP (K-sensitive) is the same as the rate of ATP-hydrolysis - disregarding whatever small spontaneous $E_a{\sim}P$ dephosphorylation there might be. Traditionally it is assumed that ADP-sensitive EP does not react with K^+ and this ion should therefore not influence the conversion step. If we consider the experimental evidence on the conversion rates of $E_a{\sim}P$ to E_bP, determined in the absence of K^+, it appears that it is fast enough for this step to be part of the Na,K-ATPase cycle. In our simulation we found k_A for ox brain enzyme at 0^oC to be rising from 0.7 s^{-1} to about 1.3 s^{-1} with increasing Na^+ (Nørby et al., 1983, Fig. 6). A similar magnitude of k_A may be deduced from the experiments by Post et al. (1969) who measured the transient formation of $E^{32}P$ from an

enzyme-$AT^{32}P$-Na^+ complex in the presence of ADP and 16 mM NaCl. It was shown that ADP, even at very high concentration (4 mM) was not able to hinder formation of $E^{32}P$. Experiments in our laboratory (Klodos, unpublished) have confirmed and extended Post's observations: $E^{32}P$ is produced in the presence of 2.5 mM ADP, but its amount decreases with increasing Na^+ and all the EP is K-sensitive. (When Na^+ is increased , k_{-B} increases and the net rate of E_bP production decreases. ADP then prevent formation of K-sensitive EP). There is thus good reason to assume that the rate of $E_a{\sim}P \longrightarrow E_bP$ (rate constant k_A, see Figure 3) is comparable to that of $E_a{\sim}P$ + ADP $\longrightarrow$ even when ADP is in mM concentrations. An estimate of k_A on this basis gives a value greater than 1 s^{-1}. Likewise Hobbs et al. (1985) have found that steady-state proportions, of what from their analysis appears to be different EP-species, is reached rapidly, pointing towards rapid rates of interconversion.

The results in the preceeding paragraph were obtained in the absence of K^+, albeit K^+ may have been used as a tool for measuring steady-state pool fractions. It is when one attempts to measure the conversion rate of ADP-sensitive to K-sensitive EP more directly, that the problems of kinetic competence arise. Whether one considers a two-pool or a three-pool model, the rate coefficient for $E_a{\sim}P$ hydrolysis - directly and/or via E_bP - should be directly measurable as the slope of the slow phase in K-dephosphorylation curves as outlined in Figure 1 and 3. It should be noted (see Nørby et al., 1983, pp. 745-746) that all K-dephosphorylation curves from EP formed in the presence of Na^+ alone exhibit this slow phase with k around 0.1 s^{-1}, but only the few studies cited below seem to allow direct comparison with measured ATP-hydrolysis under the exactly same conditions.

The value for k_A thus estimated in the presence of K^+ is at least one order of magnitude lower than that found in the absence of K^+, and the same discrepancy is seen by Hobbs et al. (1985). When they resolve their K-dephosphorylation curves (enzyme phosphorylated in the presence of Na^+ only) to determine pool sizes and rate coefficients for their hydrolysis and interconversion, they make two observations: Firstly, as discussed above, that equilibration of the pools in steady-state (with no K^+) is rapid, and secondly that the rate coefficient for the interchange between these pools, deduced from the dephosphorylation curves with K^+, is much too small to explain the rapid equilibration.

We have found for ox brain at 0°C that the rate of Na,K-ATPase activity, calculated as the product of the rate

coefficient determined in the presence of K^+ and the steady-state concentration of E_a~P (or E_1P), is smaller than the measured rate under the same conditions (Klodos and Nørby, 1979; Nørby et al., 1983), and J. C. Skou (personal communication) encounters the same problem with phosphoenzymes from shark rectal gland at 10°C. Mårdh (1975) has published a similar experiment for ox brain at 21°C (his Fig. 3C and 4A). The rate coefficient for the slow phase of his K-dephosphorylation curve can be estimated to about 47 s^{-1} which is a factor of 2 lower than that needed in the Na,K-ATPase reaction cycle.

To state the paradox (see also Glynn, this volume for a discussion): The rate constant for conversion of ADP-sensitive EP, formed in the presence of Na^+ alone, to K-sensitive EP, is much smaller in the presence of K^+ than in the absence of K^+. We have proposed that to explain this, one must assume that K^+ reacts with E_a~P (E_1P) and blocks or slows down its effective conversion to K-sensitive EP (Nørby et al., 1983). Recently further support for such an effect of K^+ on the E_a~P conversion has been obtained by Klodos (this volume). When ADP with or without K^+ was added to EP preformed in the absence of K^+, a K-stimulated ATP synthesis ("ATP burst") was observed and this happens only under conditions where a K^+-blockage of E_a~P conversion would be expected to produce such a result.

One might think of several other theoretical and experimental complications that could obscure the real reason for the observed paradox. One type of artefact that has been suggested, is that the ATP - in mM concentrations - added to dilute $AT^{32}P$ and thereby initiate dephosphorylation of $E^{32}P$, might be responsible (in combination with K^+?) for the slow phase with K^+. There is quite compelling evidence that ATP with low affinity may bind to EP (Askari, 1987; this volume). In our laboratory, however, we have been unable to find any significant effect of ATP relative to EDTA on the interconversion and dephosphorylation of ox brain Na,K-ATPase, except perhaps for a marginal inhibition of the spontaneous dephosphorylation of ADP-insensitive EP (Klodos and Nørby, this volume). In fact there are other published dephosphorylation experiments showing that chasing with mM concentration of ATP or chelation of Mg with EDTA or CDTA gives the same result when there is Na^+ and $MgCl_2$ present (Figure 2, this paper; Froehlich et al., 1983; Fukushima et al., 1984). From the paper of Klodos and Nørby (this volume) it appears that two earlier studies that have demonstrated an effect of ATP on EP under certain conditions, are not

relevant for the present discussion.

An analysis of a number of other alternative suggestions regarding the slow phase in the K-dephosphorylation curves, show that they will not explain the observation (Nørby, 1985). At present we are then left with the conclusion, that E_a~P formed in the presence of Na^+ alone is incompetent as an intermediate in the Na,K-ATPase reaction because its conversion to E_bP will be blocked or slowed down when K^+ is added.

There is, however, evidence (presented at this conference by B. Forbush III) that also this hypothesis will have to be modified soon. Forbush phosphorylated kidney enzyme with $AT^{32}P$ in the presence of 1 M Na^+. When he measured dephosphorylation of $E^{32}P$ by chasing with unlabelled ATP and K^+ he found, as expected, a low proportion of K-sensitive EP, but, if he at the point of addition of the chase also diluted the medium to decrease the concentration of Na^+ to 100 mM, a much larger proportion of EP dephosphorylated rapidly. The phosphoenzymes could be said "to forget" the high Na^+-concentration and redistribute rapidly to a composition compatible with the low Na^+-concentration. During this fall, the same type of experiments in our laboratory - with ox brain enzyme - not only confirmed Forbush's finding but furthermore showed, that the rapid redistribution and dephosphorylation also took place when the dilution was undertaken 3 seconds after the addition of K^+, i.e. during the slow phase of dephosphorylation (see Figure 2). This cannot easily be explained by a model where K^+ blocks the conversion of ADP-sensitive to K-sensitive EP.

Phosphorylation and Dephosphorylation in the Presence of both Na^+ and K^+

The steady-state level of EP in the presence of both Na^+ and K^+ is always lower than that in the presence of Na^+ alone (which, as mentioned earlier, is close to 100% of total enzyme) and it increases with ATP over a broader range. In several studies steady-state EP levels of 10 to 40% of E(total), depending upon ionic conditions and source of enzyme, have been reported (Kanazawa et al., 1967; Mårdh and Zetterquist, 1974; Mårdh 1975, 1975a; Jørgensen and Klodos, 1978; Klodos and Nørby, 1979 ; Hobbs et al., 1980; Froehlich et al., 1983; Taniguchi et al., 1984; Klodos, unpublished data with ox brain enzyme 0°C, 25 μM ATP, 300 mM Na^+ and up to 200 mM K^+; J. C. Skou, personal communication, 10°C, enzyme from shark rectal gland). It is logical

to state that EP formed in the presence of $Na^+ + K^+$ must be predominantly K-insensitive (ADP-sensitive) since K^+, which is present all the time, increases the turnover of K-sensitive EP. Mårdh (1975), Klodos and Nørby (1979), Froehlich et al. (1983) and J. C. Skou (personal communication) have accordingly found that ADP increases the rate of disappearance of the EP.

The conclusion of the previous section was that ADP-sensitive $E_a{\sim}P$ (E_1P) formed with Na^+ alone in the medium, upon addition of K^+ turned over too slowly for it to be kinetically competent in the Na,K-ATPase cycle. We may now ask whether the phosphoenzyme(s) produced by phosphorylation in the presence of both Na^+ and K^+ are kinetically competent? The simplest way to answer this question experimentally would be to stop phosphorylation by $AT^{32}P$ with unlabelled ATP or a Mg-chelator, and then compare the rate of $E^{32}P$ disappearance with the rate of ATP-hydrolysis measured under exactly the same conditions. To our knowledge only one such direct comparison has been published, namely the well-known experiment by Mårdh (1975). He finds a close agreement between the dephosphorylation rate constant ($77\ s^{-1} = 4600\ min^{-1}$) times the steady-state EP level and hydrolysis rate confirming the hypothesis that the EP formed in the presence of $Na^+ + K^+$ is kinetically competent in the Na,K-ATPase reaction.

In other cases a complex pattern of dephosphorylation after addition of ATP or EDTA has been observed both at room temperature and at 0^oC (Hobbs et al., 1980; Froehlich et al., 1983; Klodos, unpublished J. C. Skou, personal communication). In these studies at least two exponentials (and in some experiments a third) are required for satisfactory curvefitting, and further attempts to use this information to prove or disprove models for the Na,K-ATPase reaction have been unsuccesful (Mårdh, 1975; Froehlich et al., 1983). Also the "ADP-dephosphorylation" curves have several phase (Froehlich et al., 1983) confirming the suspicion that the EP formed with Na^+ and K^+ consists of more than one species.

The characteristics and the interpretation of such dephosphorylation curves is quite probably complicated by the presence of $EAT^{32}P$ in steady-state. Since EP only constitutes 10-40% of E(total) there is ample room for a significant concentration of $EAT^{32}P$ (contrary to the situation without K^+ in steady-state where EP is close to 100 %). Under these conditions the observed rate coefficient of EP-decay may of course be considerably lower that the rate constant for conversion of the ADP-sensitive to the K-

sensitive phosphoenzyme and evaluation of the kinetic competence is then by no means straight-forward.

PROBLEMS AND PERSPECTIVES

It is clearly apparent from the above that further research on the phosphoenzymes is needed before we can get a detailed picture of their function in the catalytic reaction and in transport. There are at the moment several unsolved problems. One reflection of this is the rather great diversity of models describing the release of Na^+ from the different EP's to the extracellular phase, the interaction of Na^+ with the extracellular sites in Na:Na exchange (ADP-stimulated or accompanied by ATP-hydrolysis) and the role of Na^+ in determining the proportions of the different EP species (Hara and Nakao, 1981;; Klodos et al., 1981; Nørby et al., 1983; Yoda and Yoda, 1987; Cornelius and Skou, 1985 and this volume; Pedemonte, this volume; Robinson, this volume). The reversible deocclusion and release of Na^+ to the outward facing aspects of the enzyme may be seen as a chain of events leading from $(Na_3)E_a{\sim}P$ to the E_2 dephosphoform. If we break this chain of events down into unitary processes, there logically appear at least four EP-species or EP-pools:

$$\rightleftharpoons (Na_3)E_a{\sim}P \rightleftharpoons (Na_3)E_aP \rightleftharpoons Na_{o,0-3}E_bP \rightleftharpoons Na_{o,0-3}E_cP \rightarrow$$

In this formulation $Na_{o,0-3}E_bP$ and $Na_{o,0-3}E_cP$ both represent equilibrium pools of EP with different occupancies with Na^+ on the outward-facing, low affinity sites. We show this scheme to illustrate the great theoretical diversity as regards the possible number of EP-species involved: A "blow up" of this scheme has at least 10 different EP-forms (and we have then neglected the interaction with K^+ as well as possible allosteric, regulatory or signal sites for the cations).

New approaches, like the charge transfer experiments of Fendler et al. (1985), Apell et al. (1987) and Nagel et al. (1987) that exploit the apparent electrogenic nature of the outward directed Na-transport will provide us with valuable and critical information on these steps. Likewise, detailed studies of Na^+-flux and the sidedness of cation action in well characterized reconstituted systems (e.g. Cornelius and Skou, this volume) and rapid monitoring of transmembrane Na^+ transfer in vesicles (Forbush, 1984) will be crucial for our understanding of the system.

Maybe we are only one step behind the final answer.

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POOLS OF (Na^+,K^+)-ATPase ISOFORMS IN UNBORN AND ADULT RATS CAN BE DETECTED BY THEIR SENSITIVITY TO STROPHANTHIDIN, BUT HAVE SIMILAR REACTIVITY TO Na, K AND ATP

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INTRODUCTION

It is known that (Na^+,K^+)-ATPase activity is related to cellular growth (Smith and Rozengurt, 1978) and can influence differentiation of cells (Harold, 1977). Nevertheless, the experimental evidence regarding the behavior of the sodium pump during cellular development is limited and conflicting.

Studies performed on nervous tissue show that there is an important age-related increase in the specific activity of the enzyme (Bertoni and Siegel, 1978; Atterwill et al., 1984). However, there is no agreement about some properties of (Na^+,K^+)-ATPase in the course of cellular maturation; thus, whereas some reported only changes in the number of "pump units" at different stages of rat brain development (Bertoni and Siegel, 1978), others have found that (Na^+,K^+)-ATPase preparations from the same source have differential kinetic responses to KCl and ATP (Wilson, 1979). In addition, it has been demonstrated that (Na^+,K^+)-ATPase from whole brain of fetal and postnatal rats display unequal dose-response curves for ouabain inhibition, the enzyme of fetuses being less sensitive to the inhibitor (Matsuda et al., 1984). Differences in affinity for cardiac glycosides reflects the existence of different molecular forms of the enzyme; two isotypes of (Na^+,K^+)-ATPase, containing α and $\alpha+$ subunits were first reported by Sweadner (1979).

We have investigated the relationships between some characteristics of the (Na^+,K^+)-ATPase and the maturation of nervous cells in the hippocampal formation of the rat. The

hippocampus of 19 day old rat embryos and 6 month adult rats was chosen because its anatomical and histological developmental pattern is well known (Barlett and Banker, 1977). In 10 day old embryos the characteristic shape of the hippocampus is already evident. By that time most of the neuronal cells are postmitotic and while some are migrating, others have reached their final position. Although the latter include neurons that have initiated neurite formation, they are still very immature, since neither dendritic arborization nor synapses are present. This developmental stage differs significantly from that observed in the postnatal or adult hippocampus, allowing a comparison to be made between immature cells and their mature counterparts. (Na^+,K^+)-ATPase preparations from these sources were studied in order to examine their reactivity towards Na, K and ATP, as well as their response to strophanthidin.

METHODS

Hippocampi from 6 month rats (250-300 g.) and 19 day old fetuses, killed by decapitation, were dissected according to Banker and Cowan (1977). They were placed on ice-cold 320 mM sucrose, 1 mM EDTA, 30 mM histidine (pH 7.4 at 20°C) and stored at -85 °C until used. Homogenation was carried out in 1/10 (w/v) of the storage solution with a motor driven teflon-glass homogenizer. The resulting suspension was centrifuged twice for 10 minutes at 9,000 x g; pooled supernatants were further centrifuged for 60 minutes at 30,000 x g. The final pellets were suspended in the same solution; this constituted the "crude microsomal fraction" which was used for some experiments and as the starting material for partial purification of the enzyme according to Forbush (1985). Usual specific activities of the purified enzyme were 3 μmoles of $Pi \cdot mg^{-1}$ for adult rats and 0.5 μmoles of $Pi \cdot mg^{-1}$ for fetuses; the ouabain sensitive fraction was of more than 95% and 85%, respectively. The activities for crude microsomes of adult and fetuses were 0.7 μmol of $Pi \cdot mg^{-1} \cdot min^{-1}$ and 0.1 μmol of $Pi \cdot mg^{-1} \cdot min^{-1}$; 60% and 30% respectively was ouabain sensitive. Different preparations were stored at -85° C; storage solutions for partially purified enzyme contained 25 mM imidazole (pH 7.5 at 20° C), 1 mM EDTA and 250 mM sucrose.

ATPase activity was assayed from the colorimetric determination of Pi by the method of Fiske and SubbaRow (1925) using the amidol reagent or from the release of ^{32}Pi from $(\gamma\text{-}^{32}P)$-ATP by extraction with isobutanol-benzene (Beaugé and Campos,

1983). (γ-^{32}P)-ATP was labelled according to Glynn and Chappel (1964). (Na^+,K^+)-ATPase activity was estimated as the difference of ATP hydrolysis found in the absence and presence of 1mM ouabain. Total ATP hydrolyzed was always less than 10%. Enzyme phosphorylation from (γ-^{32}P)-ATP was done according to Beaugé and Campos (1983). Protein was determined by the method of Markwell et al. (1978), using bovine serum albumin as standard. ATP (vanadium free), bovine serum albumin, ouabain, strophanthidin, Tris and imidazole were purchased from Sigma Chemical Co. USA. All other chemicals were analytical reagent grade. Radioactive Pi was obtained from the Comisión Nacional de Energía Atómica of Argentina.

RESULTS

The four preparations used were: microsomes from adult (AM) and fetuses (FM) and purified enzyme from the both sources (AE and FE respectively).

(Na^+,K^+)-ATPase ativities per mg protein under optimal ligands concentrations, as well as maximal Na dependent enzyme phosphorylation are shown in Table 1. During postnatal hippocampal development (Na^+,K^+)-ATPase activity, measured both in crude microsomes and purified enzyme, increases between six and sevenfold.

The levels of phosphoprotein formed varied following the sequence: AE>AM>FE>FM; these values correlated with those of ATPase activities in such a way that, assuming they express the density of pump units, no significant difference could be detected in the turnover number of the enzyme along the groups tested.

To determine whether the affinity of (Na^+,K^+)-ATPase for physiological ligands changes with maturation, activation curves by Na, K and ATP were perfomed in adult and fetal preparations. Although the maximal velocities were different (see also Table 1), apparent affinities (K 0.5 values) and Hill coeficients were similar for each ligand (see Table 2 for NaCl and KCl) in all groups.

In the case of ATP, activation kinetics were explored in the milimolar and the micromolar range. Table 3 indicates that the Km within each range were not significantly different in the four groups studied.

TABLE 1. Maximal (Na^+,K^+)-ATPase activity, Na dependent phosphorylation and turnover number of partially purified (Na^+,K^+)-ATPase from adult (AE) and fetal (FE) rats, and crude microsomal (Na^+,K^+)-ATPase from adult (AM) and fetuses (FM).

Enzyme preparation	(Na^+,K^+)-ATPase activity (μmol Pi·mg^{-1}·min^{-1})	Phosphorylated enzyme (nmol Pi·mg^{-1})	Turnover number min^{-1}
AE	2.98 ± 0.011	0.28 ± 0.006	10,541 ± 1833
AM	0.73 ± 0.005	0.07 ± 0.010	9865 ± 540
FE	0.46 ± 0.007	0.05 ± 0.005	9256 ± 1320
FM	0.10 ± 0.001	0.01 ± 0.001	9191 ± 125

Phosphorylation was carried out for 10s at 0°C in media containing: 30 mM imidazole (pH 7.4 at 20° C), 1 mM $MgCl_2$ and 0.2 mM EGTA. Na dependent phosphorylation is the difference between values obtained in the presence of 130 mM NaCl and 130 mM KCl. ATPase activity was determined with the amidol reagent in a medium containing: 3 mM $MgCl_2$, 3 mM ATP, 30 mM imidazole (pH 7.4 at 20°C), 20 mM KCl, 130 mM NaCl and 0.2 mM EGTA. Each value is the mean ± SEM of triplicate determinations.

Dose-response curves for strophanthidin inhibition were performed with partially purified (Na^+,K^+)-ATPase from adult and fetal hippocampus. The experimental points, which showed an heterogeneous response, were adjusted by a non linear regression computing program to two equations. In one of them, the existence of two enzyme isoforms with different affinity for the inhibitor was assumed; in the other three isoforms were considered. The fitting achieved with the two enzyme population model gave the following distribution: 79% with high affinity and 21% with low affinity in the adult and 46% and 54% for the fetal enzyme. The Ki for the high affinity component was 2.36×10^{-7} M for adult and 2.90×10^{-7} M for fetuses; the Ki for the low affinity sites was 9.96×10^{-4} M and 7.14×10^{-4} respectively. A fitting with a three component model turned out to be more accurate. Three different populations with an affinity of 1.41×10^{-9}M, 4.45×10^{-7} M and 1.87×10^{-3} M resulted from the

TABLE 2. Apparent affinities (K 0.5) and Hill coefficients for NaCl and KCl activation of adult and fetal hippocampal (Na^+,K^+)-ATPase.

Enzyme preparation	NaCl activation K 0.5 (mM)	nH	KCl activation K 0.5 (mM)	nH
AE	16.2 ± 0.6	1.80 ± 0.04	1.77 ± 0.09	1.68 ± 0.04
FE	15.8 ± 1.4	1.86 ± 0.04	1.93 ± 0.15	1.70 ± 0.03
AM	17.6 ± 1.0	1.78 ± 0.08	1.70 ± 0.15	1.73 ± 0.19
FM	16.0 ± 0.6	1.83 ± 0.03	2.20 ± 0.03	1.56 ± 0.04

ATPase assays were carried out with the amidol reagent, using 30 min. incubation at 37°C, in a medium containing 3 mM $MgCl_2$, 3mM ATP, 0.2 mM EGTA and Tris-HCl pH (20°C) 7.4. NaCl activation was tested at 20 mM KCl, while KCl activation was performed at 130 mM NaCl. Ionic strengh was kept constant at 180 mM with Tris-HCl. The results are the mean ± SEM of three determinations.

Table 3. Km values for ATP activation of adult and fetal rat (Na^+,K^+)-ATPase activities.

Enzyme preparation	Km for ATP Low affinity (mM)	High affinity (µM)
AE	0.16 ± 0.02	0.54 ± 0.10
FE	0.20 ± 0.05	0.65 ± 0.13
AM	0.19 ± 0.04	0.39 ± 0.19
FM	0.23 ± 0.05	0.69 ± 0.16

ATPase activity was determined following the relase of ^{32}Pi from γ-^{32}P-ATP. The assay medium contained NaCl 120 mM, KCl 30 mM, $MgCl_2$ 1 mM, EGTA 0.2 mM, Tris-Cl 30 mM (pH 7.4 at 37°C) and variable concentrations of ATP. Each value is the mean ± SEM of triplicate determinations.

interaction of the adult enzyme with strophanthidin. The fractional concentration of each isoform was 13.8%, 63.7% and 22.5 %, respectively. For the fetal enzyme the Ki were 6.90 x 10^{-8} M, 5.87 x 10^{-7} M and 9.11 x 10^{-4} M; with a relative amount of 13.5%, 32.2% and 54.2%, respectively (Fig. 1).

DISCUSSION

Partially purified preparations of membrane bound (Na^+,K^+)-ATPase, as well as crude microsomes showed an increase in (Na^+,K^+)-ATPase activity with the maturation of the

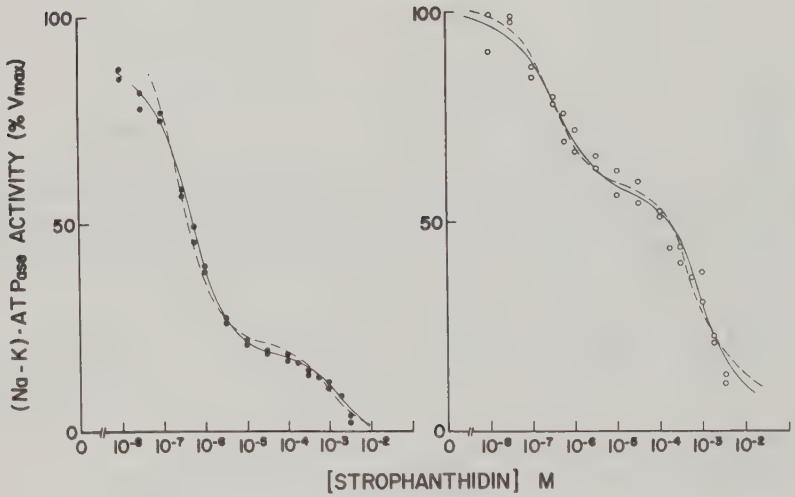

Figure 1. Strophanthidin inhibition of partially purified (Na^+,K^+)-ATPase from adult (•) and fetal (o) hippocampus. ATPase activity was determined following the release of ^{32}Pi from (γ-^{32}P)-ATP. Samples were preincubated for 20 minutes at 37°C in an incubation medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4 at 37°C), and variable concentrations of the inhibitor; the reaction was started with the addition of ATP (final concentration 3 mM). Each point is the mean of duplicate determinations. The curves are the best fits corresponding to heterogeneous enzyme populations of two (---) or three (—) isoforms with different sensitivity to strophanthidin.

hippocampal formation. This increment cannot be attributed to changes in the properties of the enzyme with development, since no consistent changes in the turnover number or in the dependence to physiological ligands occur with hippocampic growth. Increased activity can rather be explained by an increment in the density of enzyme molecules at the membranes of mature cells.

Different forms of (Na^+,K^+)-ATPase could be identified on the basis of their sensitivity to strophanthidin; although there was a mixture of the isoforms in both adult and fetal preparations, their relative amounts varied with maturation. This might indicate that the relative proportions of the (Na^+,K^+)-ATPase isoforms in neuronal cells depends on the moments of their development. Since the fundamental event in neuronal maturation is the formation of processes and synapses, it might be conceivable that different isoforms predominate in different parts of the same cell. However, it cannot be excluded that cells other than neurons could account for the present results.

The fact that a similar response towards Na, K and ATP was observed in the four preparations is consistent with the idea that (Na^+,K^+)-ATPase isoforms have the same reactivity towards the ligands.

Finally, the lack of differences between the kinetic parameters studied in crude microsomes and partially purified enzymes would argue against the existence of regulatory membrane proteins of the sodium pump removable by SDS in both immature and mature cells.

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MECHANISM OF SUBSTRATE SPECIFICITY OF THE Na,K-PUMP

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INTRODUCTION

It is a well known fact that Na,K-ATPase does not possess an absolute specificity toward the substrate ATP: in addition to ATP it hydrolyses other nucleotides, p-nitrophenyl phosphate, acetyl phosphate, umbelliferone phosphate and so on. In all cases a phosphorylated intermediate, EP, is the formed and the hydrolytic reaction is inhibited by the specific Na,K-ATPase inhibitor, ouabain. The energy liberated from hydrolysis of all nucleotides is enough for active transport of ions, however, the Na,K-pump works only during ATP or CTP hydrolysis (Svinukhova and Boldyrev, 1987). The aim of our investigation was to elucidate how ATP and CTP couple hydrolytic and transport functions of the Na,K-pump.

RESULTS AND DISCUSSION

Na,K-ATPase from two sources was used in these experiments: from ox brain with specific activity of 2-3 μmoles ATP split/mg protein per min (Klodos et al., 1975) and from duck salt glands with specific activity of 20-25 μmoles ATP split/mg protein per min (Boldyrev et al., 1981).

All of the substrates utilized by the enzyme are divided in three groups. ATP and CTP belong to group I: concentration dependence of their hydrolysis is expressed as a curve with an interjacent plateau and does not follow Michaelis - Menten kinetics; for both of them two K_m values can be calculated corresponding to two types of nucleotide binding sites with high and low affinity (Table I).

GTP and UTP represent substrates of group II: they are hydrolysed in accordance with hyperbolic kinetics, having a single K_m value; V_m for them are essentially lower than that for ATP. ITP has an intermediate position: being close to ATP structure, this nucleotide usually shows a simple hyperbolic behaviour of the hydrolysis (Table I). At the same time a precise analysis makes it possible to identify a deviation from Michaelis - Menten kinetics which, however, is much less pronounced than that for ATP (Boldyrev and Svinukhova, 1982).

TABLE I. Kinetic parameters of duck salt gland Na,K-ATPase with different nucleotides and umbelliferone phosphate

Substrate	V_m (μmoles P_i/mg protein per min)	K_m (μM)
ATP	13.3 and 33.3	10 and 330
CTP	2.7 and 6.7	20 and 400
ITP	3.3	200
GTP	1.8	650
UTP	1.4	2000
Umbelliferone phosphate	1.4	400

Na^+ and K^+ activate hydrolysis of different nucleotides in a diverse manner (Skou, 1974). For brain enzyme 50% activation of hydrolytic activity is provided by Na/K (mM/mM): 40/110 (with ATP), 50/100 (with CTP), 70/80 (with ITP, 100/50 (with GTP). Thus hydrolysis of all the nucleotides is activated by Na^+ and K^+ jointly, but substrates of group II have a relatively lower affinity to Na^+.

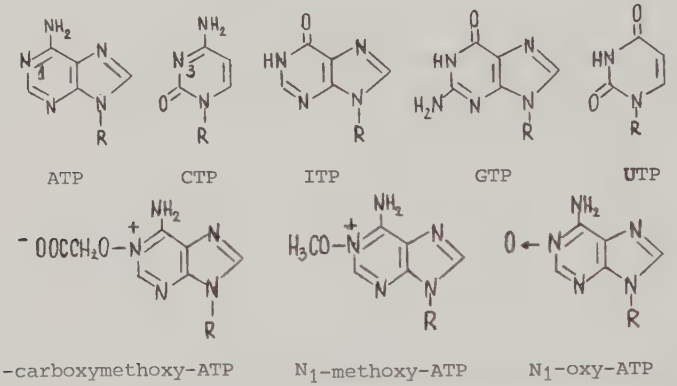
Non-nucleotidic substrates represent group III. They provide the so called K-activated phosphatase reaction. Like group II substrates they follow Michaelis - Menten kinetics,

but possess higher K_m and lower V_m. In contrast to groups I and II substrates they are hydrolysed with the higher rate in the absence of Na^+, which inhibits their splitting. Addition of the substrate of groups I or II removes an inhibiting effect of Na^+ on K-phosphatase reaction. This is explained by a generally accepted scheme of Na,K-ATPase operation. According to Cantley et al. (1983) ATP hydrolysis is performed during transformation of E_1 (Na-form) to E_2 (K-form), the rate limiting step being $E_2 \rightarrow E_1$ transition. Acceleration of the reaction by an increase in the ATP concentration is explained by facilitation of the limiting step as the result of the interaction of ATP with low affinity site. Hydrolysis of group III substrates is provided by K-form of the enzyme and Na^+ inhibition is due to transformation of part of molecules into Na-form (E_1). ATP added returns them during the hydrolytic cycle into the E_2 conformation, which can utilize group III substrates. Thus, ATP activates the phosphatase reaction during its own hydrolysis and exhaustion of ATP stops activation. As $E_2 \rightarrow E_1$ transition is a limiting step of the ATPase hydrolytic cycle, activation of phosphatase reaction (measured in the presence of Na^+ and K^+) by nucleotide characterizes indirectly its ability to facilitate $E_2 \rightarrow E_1$ transformation.

Comparison of the structure of different nucleotides shows that group I substrates have a common property: under physiological pH values N_1 atom of the purine base (N_3 atom of the pyrimidine base) is deprotonated and can form hydrogen bond with an appropriate group in the active center of the enzyme. Group II substrates are protonated at pH 7.4 - 7.8 and do not possess proton binding capacity. ITP (pK_a = 8.8) has an intermediate position, with part of molecules being deprotonated at physiological pH.

As Skou (1982) proposed, the $E_2 \rightarrow E_1$ transition is followed by deprotonation of a definite group (suggesting histidine-13; Jorgensen and Collins, 1986) in the active center of the enzyme. With this in mind we proposed, that acceleration of $E_2 \rightarrow E_1$ transition after interaction of ATP (CTP) with the enzyme is explained by hydrogen bond formation between the N_1 (N_3) atom of the nucleotide base and an appropriate group on the enzyme (Boldyrev, 1985). To verify this hypothesis we have investigated the effect of a number of nucleotides with different proton-accepting capacity on the $E_2 \rightarrow E_1$ transition and have compared these data with the ability of the nucleotides to provide for active $^{22}Na^+$ transport. Besides natural nucleotides, synthetic ATP analogs, containing negatively charged substituents in the base

(N_1-oxy-ATP and N_1-carboxymethoxy-ATP) and an analog, containing an uncharged substituent (N_1-methoxy-ATP) were used. Such a difference in structure suggests high proton-accepting capacity of two former ATP analogs comparing to that of the latter one.



While comparing the influence of the nucleotides on the umbelliferone phosphate hydrolysis by Na,K-ATPase in the simultaneous presence of Na^+ and K^+, we could see that group I substrates are better activators of K-phosphatase, their effect being the result of the interaction with a high affinity site (see $K_{0.5}$ values in Table II). Group II substrates activate K-phosphatase to a smaller extent, whereas the activating effect of synthetic ATP analogs correlates with their proton-accepting capacity.

The same regularity was found in the experiments where $E_2 \rightarrow E_1$ transition was monitored by measuring changes in the fluorescense of enzyme labeled with 5-iodoacetamido-fluorescein (Svinukhova et al., 1987). Fluorescence intensity was decreased after K^+ addition and could be restored after addition of Na^+ or nucleotides. When the $E_2 \rightarrow E_1$ shift was measured in the presence of 10 μM KCl, a fluorescence level corresponding to the Na-form of the enzyme was achieved at ATP concentrations of 50-100 μM. CTP and ITP provided for this effect at higher concentration, but GTP and UTP failed to give such a fluorescense level even at 10 mM (Fig. 1A). In the presence of 40 μM KCl more pronounced difference between substrates of different groups was found (Fig. 1B. In other words, the ability of nucleotides to facilitate $E_2 \rightarrow E_1$ transition is in a good correlation with their proton-accepting properties: ATP>CTP>ITP>GTP = UTP. Synthetic ATP analogs behave in a predicted manner: N_1-methoxy-ATP like GTP, and N_1-oxy-ATP like CTP (not shown).

Using duck salt gland Na,K-ATPase reconstituted into phospholipid liposomes we could measure ouabain dependent active $^{22}Na^{+}$ transport (Svinukhova and Boldyrev, 1987).

TABLE II. Activating effect of nucleotides on umbelliferone phosphate hydrolysis by Na,K-ATPase from duck salt gland.

NTP	Activating effect (% of control)	$K_{0.5}$ (μM)
ATP	1700	10
N_1-carboxymethoxy-ATP	1700	20
N_1-oxy-ATP	1700	30
CTP	1500	100
ITP	1400	200
UTP	500	1000
GTP	120	2000
N_1-methoxy-ATP	No activation	

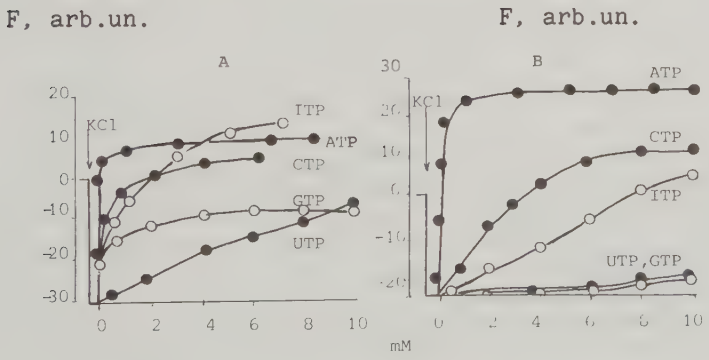


Fig. 1. Effect of nucleotides on the fluorescense of 5-IAF-labeled Na,K-ATPase from duck salt gland in the presence of 10 μM KCl (A) and 40 μM KCl (B).

We have found the enzyme transport function to be in a strong correlation with the ability of substrates to modify the affinity of Na,K-ATPase to Na^+ and K^+ (in other words, to facilitate transformation of K-form into Na-form). At Na/K ratio of 20 mM/50mM (Fig. 2A) group I substrates are hydrolyzed in a coupled manner, providing for an active $^{22}Na^+$ accumulation. N_1-oxy-ATP provides for the same regime of the enzyme operation. Group II substrates cannot provide for transport function under conditions used. ITP and N_1-methoxy-ATP occupy an intermediate position. The ability of ITP to provide for active Na_+ transport can be amplified by an increase in Na^+ and a decrease in the K^+ concentration (at a Na/K ratio of 50 mM/20 mM). However, even under these conditions neither UTP nor GTP could provide for Na/K-pump operation (Fig. 2B).

The data presented are in agreement with the conception of Cantley et al. (1983). According to this conception Na,K-ATPase can operate in a protomeric ($\alpha\beta$) form, and high and low affinity sites for substrate belong to different conformations (Na- and K-form) of the protomer. However, a number of facts are in contradiction with such a picture. Thus, Ottolenghi and Jensen (1983) have found Scatchard plots for ATP binding by pig kidney enzyme (detergent treated) to be linear in the presence of Na^+, but non-linear after K^+ addition. Duck salt gland enzyme is characterized by the heterogeneity of ATP binding sites both in Na- and in K-containing media (Altstein et al., 1986). This feature was shown also for duck salt glands microsomes, which were not treated with detergent.

Calculation of K_d values for high and low affinity sites gives two distinct values (0.2 and 3.0 μM), with ratio between sites of high and low affinity being equal to 1:3 (Altstein et al., 1986). Binding study of other ligands also supports the idea of interaction of protomers during enzyme functioning (Hansen et al., 1979).

We have examined this possibility using radiation inactivation analysis. As seen from Fig. 3, the rate of enzyme inactivation, which, in general, is proportional to the target size, depends on the substrate nature. For both enzyme preparations (from ox brain and from duck salt glands) the molecular complex hydrolysing ATP or CTP (group I substrates) is twice as large as that hydrolysing GTP or p-NPP (Group II and III substrates). In all of these experiments the substrates were used at saturating concentrations. Decrease in ATP concentration up to 30 μM results in the reduction in the molecular size by a factor of 2 (not

shown). Thus, it is a saturation of low affinity sites, which is a factor essential for oligomerization of (αβ) protomers. Recently our results have found independent confirmation (Cavieres, 1987).

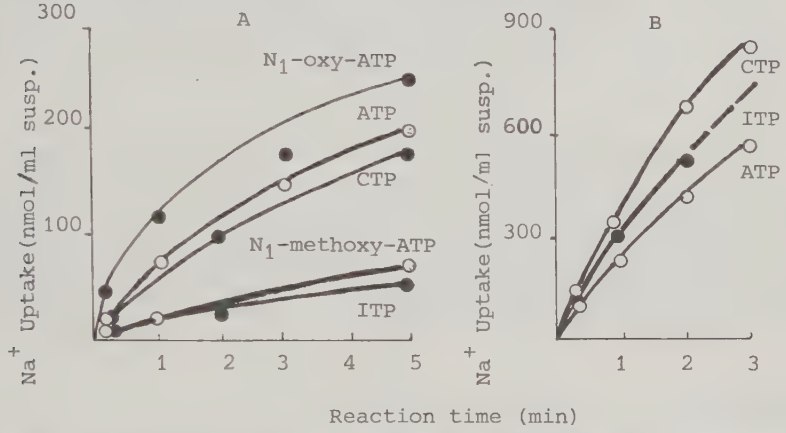


Fig. 2. Accumulation of $^{22}Na^+$ by proteoliposomes with reconstituted Na,K-ATPase in the presence of various nucleotides at Na/K ratio of 20 mM/50mM (A) or 50mM/20mM (B).

From this point of view it is interesting that Arrheinius' plots for Na,K-ATPase could be linear (with GTP, UTP or p-NPP as substrate) or could demonstrate a break (in the case of CTP or ATP at high concentrations) at the temperature where lipid bilayer rearrangement take place (Boldyrev, 1981, 1985). In this last case membrane fluidity controls ATPase activity, affecting interaction between protomers

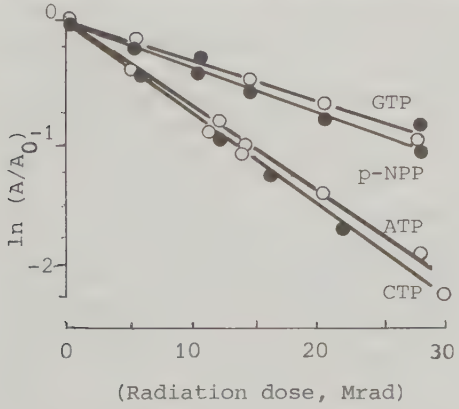


Fig. 3. The effect of increasing doses of radiation on the ability of ox brain Na,K-ATPase to hydrolyse ATP, CTP, GTP or p-NPP at saturating concentrations.

in the oligomeric complexes of the enzyme. In fact, a decrease in ATP concentration down to 30 μM, when no interactions between protomers is observed (in radiation inactivation experiments) converts a non-linear Arrhenius plot to a linear one, indistinguishable from that with GTP or p-NPP. We suggest that group I substrates interacting with low affinity sites of the enzyme induce oligomeric complexes, formation which are sensitive to the phase state of lipid matrix.

Thus oligomeric structure of Na,K-ATPase is a factor which makes it possible to regulate enzyme activity through the control of interprotein connections by lipid bilayer fluidity and occupation of low affinity sites by substrate. Both features may regulate the efficiency of transformation of chemical energy from ATP into the electrochemical potential of ionic gradients of the cell.

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ISOLATION OF TWO ISOFORMS OF NA,K-ATPASE FROM ARTEMIA SALINA: KINETIC CHARACTERIZATION

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INTRODUCTION

The larval and adult forms of the brine shrimp, e.g. Artemia salina, tolerate an ambient salinity of 50-4900 mM Na while maintaining a hemolymph concentration of 86-116 mM (Russler and Mangos, 1978; Conte, 1980). In various species that exist in widely differing salinities (e.g. lakes and oceans), ouabain at 10^{-4}M, abolishes this gradient, markedly reducing survival, as ambient salinity is increased, and ultimately leading to death (Ewing, et al., 1972). This suggested that Na,K-ATPase plays a critical role in maintaining this essential ionic balance. In Artemia salina, the specific activity of Na,K-ATPase increased with development from almost undetectable levels in the cyst to a steady state at around 42 hours. Partially purified Na,K-ATPase from whole nauplii exhibit 2 alpha subunits, alpha 1 and alpha 2, when analyzed by SDS-PAGE and identified by Na^+ and Mg^{2+} dependent gamma $^{(32)}$ATP labelling (Peterson et al., 1978). To characterize the kinetic and molecular properties of these two isoforms, and establish their functional significance, the two major transepithelial transporting organs in the brine shrimp, the salt gland and intestines had to be isolated in adequate amounts and a protocol for the purification of Na,K-ATPase from each developed. Lowy and Conte (1985) succeeded in isolating salt glands in batchwise processing and in dissecting out intestines from nauplii. The yield, however, was low and allowed for microscopic characterization and measurement of maximal rates (V_{max}) for Na,K-ATPase only in

whole homogenates. The salt gland enzyme had a V_{max} twice that of the enzyme from the intestines. In this study, we describe isolation of salt glands and intestines in large amounts and in almost pure suspensions that allowed for the kinetic characterization of two isoforms of Na,K-ATPase. FITC labelling and SDS-PAGE indicate that the salt glands express one of the isoforms while the intestines contains both isoforms.

MATERIALS AND METHODS

Brine shrimp cysts, Artemia franciscana GSL, were hydrated in distilled water at 4°C for 8-12 hrs. The hydrated cysts were then collected, washed thoroughly and transfered to full strength artificial salt water pH 9.0 and incubated for variable periods of time, usually 24 hrs at 29°C. The hatched nauplii were collected and transferred to a high phosphate solution (SGRM) as described by Lowy and Conte (1985), and the incubation was carried at 37°C for 8 hrs. The nauplii were then collected, vortexed and passed through a series of sieves. This mixture, predominantly of salt glands and intestines, was passed through a 30x2.5 cm column of beads 1 mM in diameter. The salt glands were collected in the effluent and the intestines were then eluted off the beads. Suspensions of either salt glands or intestines were collected by centrifugation at 200xG in a clinical centrifuge followed by 2 washings with sucrose-EDTA-Imidazole.

Assay of Na,K- ATPase.

Whole homogenates of the salt glands and intestinal tubes were prepared by homogenizing the tissue in 1:10 V/V of 250 mM sucrose, 2 mM EDTA, 50 mM imidazole pH 7.2 (SHM). Microsomal membranes were prepared according to Jorgensen (1974). The respective pellets were suspended in SHM to an approximate concentration of 1 mg/ml. and stored at -20°C. Na,K- ATPase was assayed by monitoring the rate of [gamma-32P] ATP hydrolysis as described by Muallem and Karlish (1980) and modified by Pressley et al (1986), in varying concentrations of Na,K and ATP for the kinetic studies.

Effect of sodium deoxycholate.

Sodium deoxycholate in concentrations ranging from 0 to 1.5 mg/ml were incubated with 100 ug of enzyme protein at pH 7.2 in a final volume of 100 microliters for 30 minutes at room temperature. At the end of the incubation 5

microliters from each tube were immediately assayed for Na,K- ATPase.

Labeling with Fluorescein Isothiocyanate (FITC).

Enzyme protein was incubated for 30 min. at room temperature with 10 uM FITC at pH 9, in the presence and absence of 5 mM ATP as described by Salon (1985). The products were analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

The isolated salt glands and intestinal tubes were checked for homogeneity by light microscopy, (Figures 1 and 2). About 80 mg of salt glands and 500 mg of intestinal

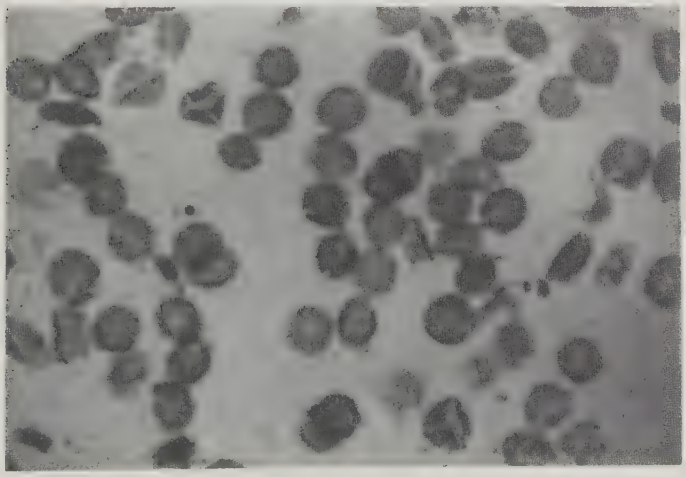

Fig. 1 Photomicrograph of a field of isolated brine shrimp salt glands (50x).

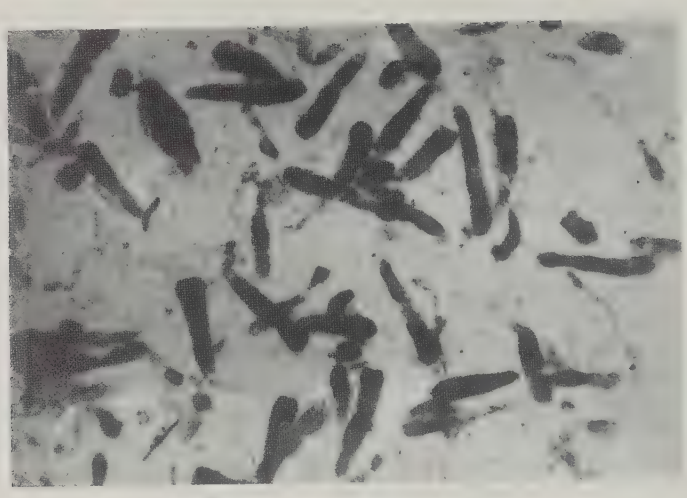

Fig. 2 Photomicrograph of a field of brine shrimp intestinal tubes (50x)

tubes were obtained from 400 g of dried brine shrimp cysts, Artemia franciscana GSL. Specific activity of Na,K-ATPase in whole homegenates was 32±6 (umoles Pi/hr/mg protein) in the salt glands and 12±3 in the intestinal tubes. The specific activity of the salt gland enzyme increased from 19 to 32 between 26 and 32 hours of development and then dropped to 16 and 10 by 40 and 50 hours of development respectively. The apparent half maximal activation constants (K1/2) of the salt gland enzyme as compared to the intestinal tube enzyme were 3.7±0.6 mM versus 23.5±4 mM ($P<0.01$) for Na respectively, 16.6±2 mM versus 8.29±1.5 mM ($P>0.01$) for K respectively and 0.87±0.8 versus 0.79±1.1 (NS) for ATP respectively. The apparent Ki's for ouabain were 10^{-4} M versus $2x10^{-5}$ M respectively. Sodium deoxycholate produced a maximal Na,K-ATPase activation of 46% in the salt gland enzyme as compared to 23% in the enzyme isolated from intestinal tubes. These findings indicate that two functional isoforms of Na,K-ATPase exist in the brine shrimp and that their relative abundance in salt glands and intestinal tubes differ. To investigate this further, partially purified enzyme from the salt glands and intestinal tubes were prepared with a Na,K-ATPase activity of 290 and 210 (umoles Pi/hr/mg protein) respectively and analyzed by SDS-PAGE (Figure 3). In the salt gland only

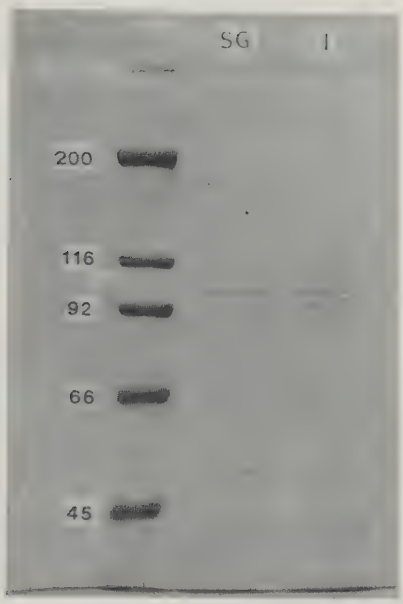

Fig. 3 Photograph of a Coomasie blue stained SDS-PAGE gel. Lanes from left to right, HMW standard, partially purified enzyme (290u) from salt glands (SG) and partially purified enzyme (210u) from intestinal tubes (I).

one isoform is evident, while the intestinal tube exhibits the two forms. This finding, in the brine shrimp, is similar to the observation of Sweadner (1979), that one form of alpha subunits was predominant in neuronal tissue, whereas another form, is predominant in the kidney medulla, as well as, in glial tissue. To eliminate the possibility of differential loss of one of the isoforms during the purification of the enzyme from the salt gland, FITC labeling was performed on whole homogenates. In these preparations only one ATP inhibitable band was seen in the salt gland and two in the intestinal tube enzyme. In conclusion, two isoforms of Na,K-ATPase were isolated from the brine shrimp that differ markedly and significantly in three kinetic parameters as well as in their sensitivity to sodium deoxycholate. The differences in the K1/2s for Na and K are more marked than those reported for mammalian Na,K-ATPase isoforms.

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DETERMINATION OF OX BRAIN Na,K-ATPase ISOFORMS AND THEIR PHOSPHORYLATED INTERMEDIATES BY ANALYSIS OF ANTHROYLOUABAIN BINDING AND ITS INHIBITION BY OLIGOMYCIN

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INTRODUCTION

It has been shown (Fortes and Lee, 1984) that the binding of the fluorescent probe anthroylouabain (AO) (Fortes, 1986) to Na,K-ATPase may be followed by monitoring the fluorescence as a function of time, and that analysis of such binding curves obtained during turnover of the enzyme may yield information about the steady state concentration of the intermediates to which AO binds. This method has been employed here in a study of the ox brain enzyme during turnover at 37°C (pH 7.4) for the following purposes: 1) Determination of the concentration of active sites, 2) Detection of (at least two) different isoenzymes, 3) Detection of (at least three) phosphorylated intermediates in each isoform, 4) Determination of the steady state concentration of E_1P during turnover at 37°C, by investigating the influence of oligomycin on the AO binding kinetics.

METHODS

Na,K-ATPase was partially purified from ox brain by the procedure of Klodos et al. (1975). The maximal phosphorylation capacity was 0.49 nmoles/mg protein.

Fluorimetric measurements of AO binding were performed as described by Fortes and Lee (1984) under pseudo-first order conditions, i.e. the AO concentration was always more than 10 times the total enzyme concentration. The AO concentration ranged from 270 nM to 2.5 μM, and the enzyme concentration ranged from 27 nM to 170 nM. All experiments were carried out with $[Mg^{2+}_{free}] = 1$ mM in 30 mM imidazole buffer, pH 7.4 at 37°C.

When desired, the ionic strength was kept constant using tetra methyl ammoniumchloride (TMA).

Conversion of fluorescence units to concentration of specifically bound AO, $[AO]_{bound}$, was performed as follows (Fortes, 1986):

$$[AO]_{bound} = \frac{\Delta F}{\phi(\gamma - \delta)}$$

where ΔF is the fluorescence change due to specific binding, ϕ is the fluorescence in the absence of enzyme divided by the AO concentration, γ is the ratio of the quantum yields of bound and free AO (determined by titration in a separate experiment) and δ is the ratio F_I/F_{free}, where F_I is the fluorescence of free + nonspecifically bound AO, and F_{free} is the fluorescence of free AO.

THEORY

The binding of AO in a dead-end fashion to a single conformation, such as E_2P:

$$E_2P + AO \rightleftarrows E_2P \cdot AO$$

with binding and release rate constants k_2 and k_r is governed by the equation

$$\frac{d[E_2P \cdot AO]}{dt} = k_2[AO][E_2P] - k_r[E_2P \cdot AO]. \qquad (1)$$

If binding and release reactions are much slower than the steady state turnover rate of the enzyme, $[E_2P]$ is at all times a constant fraction, f_2, of the total available enzyme:

$$[E_2P] = f_2([E_T] - [E_2P \cdot AO]). \qquad (2)$$

Eq. (1) then becomes:

$$\frac{d[E_2P \cdot AO]}{dt} = f_2k_2[AO][E_T] - (f_2k_2[AO] + k_r)[E_2P \cdot AO] \qquad (3)$$

whose solution is (with a large surplus of AO).

$$[E_2P \cdot AO]_t = [E_T](1 - \exp[-(f_2k_2[AO] + k_r)t]) \qquad (4)$$

The AO release reaction, observed after adding ouabain to the AO-equilibrated enzyme suspension, follows simple first order kinetics, and hence

$$\ln\left[\frac{[E_2P \cdot AO]_t}{[E_T]}\right] = -k_r t. \qquad (5)$$

Correspondingly, Eq. (3) transforms into:

$$\ln\left[1 - \frac{[E_2P \cdot AO]_t}{[E_T]}\right] = -(f_2k_2[AO] + k_r)t. \qquad (6)$$

From experiments conforming to these equations k_r and f_2k_2 can be determined.

In reality, AO binds to more than one enzyme conformation (i.e., different phosphorylated intermediates) at significant rates. Under these conditions the binding equation described above can be applied but the observed rate constant for binding is the average of the intrinsic binding rate constants k_i weighted by the fractional concentrations of the intermediates f_i:

$$k_{on} = \sum_{i=1}^{n} f_i\, k_i. \qquad (7)$$

Thus, variations in the relative concentration of the different binding conformations caused by changes in the incubation conditions will be manifested by changes in k_{on} if the intrinsic rate constants, k_i, of the conformations that bind AO are different. Therefore, measurements of k_{on} can be used to monitor redistributions of intermediate forms during steady state turnover.

Experimentally, the binding and release of AO to ox brain (Na,K)-ATPase did not follow single exponentials. A sum of two exponentials gave good fits to the data. This behavior can be explained by the existence in the preparation of two independent (Na,K)-ATPase isoforms, each participating in AO binding. We will denote the two isoforms with the superscripts $\alpha 1$ and $\alpha 2$, respectively, and the fraction of the enzyme consisting of isoform $\alpha 1$ is denoted x. If the AO release rate constants of all the different intermediates of each isoform are the same, (or if they are negligible compared to the pseudo-first order

binding constants), the kinetics of AO binding may be shown to be described by

$$(\text{total binding})_t = E_T\{1 - x\exp[-(k_{on}^{\alpha 1}\,AO + k_{off}^{\alpha 1})t] - (1-x)\exp[-(k_{on}^{\alpha 2}\,AO + k_{off}^{\alpha 2})t]\}. \quad (8)$$

If only initial rates are measured, it may be shown that

$$v_{init} = k_{av} \cdot [AO] \cdot [E_T] \quad (9)$$

where

$$k_{av} = x\,k_{on}^{\alpha 1} + (1-x)\,k_{on}^{\alpha 2}. \quad (10)$$

RESULTS

Enzyme concentration. In the presence of 300 nM AO the concentration of bound AO was 26.5 nM, compared with 27.5 nM obtained by phosphorylation with [γ-^{32}P]-ATP, i.e., the fluorimetric method gave 96% of the value obtained by phosphorylation. Other experiments with 5 times higher AO concentrations showed no increase in specific AO binding, indicating that the enzyme was saturated with 300 nM AO. This result indicates that the ox brain preparation has a high affinity for AO.

Analysis of AO binding and release. The semilogarithmic plots of the time course of AO binding or release were non-linear under all conditions tested. This is in contrast with the behavior of mammalian kidney and electrophorus (Na,K)-ATPases, which gave linear plots under the same conditions. A sum of two exponentials gave very good fits to the data. We conclude that at least two (Na,K)-ATPase isoforms participated in AO binding. From a large number of determinations the relative abundance of the two isoforms was 50 ± 20%, in agreement with determinations by SDS-page of α and α^+ content in similar preparations (K. Sweadner, personal communication). The rate constants of the fast and slow components differed by a factor of 3 to 5 for the binding reaction and a factor of 5 to 7 for the dissociation reaction. The affinities of the two isoforms for AO, calculated from the kinetic data, are very similar, with $K_D = 15 - 30$ nM, in agreement with the equilibrium binding data which indicated that 300 nM AO was saturating.

For the purpose of studying rate constant variation with ligand concentration we used initial rate measurements (Eqs. (7-8)) since k_{av} could be determined with higher accuracy than the fractional concentrations and rate constants of the individual isoforms.

Effect of Na^+. The variation of k_{av} and Na-ATPase activity with Na are shown in Fig.1. The values of k_{av} first increased and then decreased when the $[Na^+]$ was increased. The ionic strength was maintained constant at 2 M with tetramethylammonium (TMA) to minimize effects due to changes in

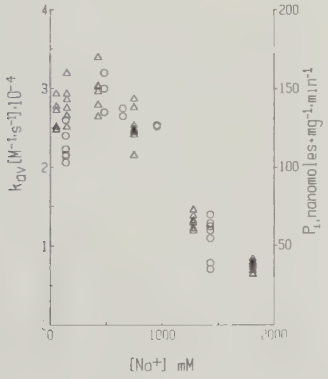

Fig. 1. The average binding rate constant for AO (O,ATP = 30 μM, left ordinate) at constant ionic strength: $[Na^+ + TMA] = 1.9$ M, and Na-ATPase activity (Δ,ATP=3 mM, right ordinate), as functions of $[Na^+]$. pH = 7.4, 37°C.

ionic strength. Increasing [ATP] from 30 μM to 3.5 mM had little effect on k_{av} (results not shown). It is noteworthy that the changes in Na^+-ATPase activity with increasing $[Na^+]$ were parallel to those in k_{av}. Both brain isoforms appeared to respond similarly to Na^+. Since most of the enzyme is phosphorylated during incubation with MgATP and Na, and the phosphorylation levels are independent of $[Na^+]$ (Lee and Fortes, 1985), the changes in the value of k_{av} when $[Na^+]$ is varied reflect redestributions of different phosphorylated intermediates that have different intrinsic AO binding rate constants, i.e., different conformations. The results with the brain preparation were essentially the same as those with the kidney (Lee and Fortes, 1985) and are consistent with at least three phosphorylated intermediates in the Na^+-ATPase reaction.

Determination of $[E_1P]$ - inhibition by oligomycin. If oligomycin binds to only one intermediate, E_1P, in a dead-end fashion, then the fractional concentration f_1 of E_1P may be determined by analyzing the effect of oligomycin on AO binding. It can be shown that in the presence of oligomycin (we assume that it binds equally well to both (Na,K)-ATPase isoforms),

the average rate constant for binding of AO is:

$$k_{Oli} = \frac{k_{av}}{1 + f_1 (\frac{[Oli]}{K_{Oli}})} \quad (11)$$

where K_{Oli} is the dissociation constant of $E_1P \cdot Oli$ to E_1P + Oli. Thus a plot of the ratio

$$k_{av}/k_{Oli} = 1 + f_1 \frac{[Oli]}{K_{Oli}} \quad (12)$$

will have a slope = f_1/K_{Oli}. Under conditions where essentially all of the enzyme is in the E_1P form, e.g. turnover in the presence of high Na and absence of K, K_{Oli} may be determined from the inhibition of AO binding by oligomycin, because $f_1 = 1$. Once K_{Oli} is known the value of f_1 at physiological Na concentrations can be obtained from the slopes of the Dixon-type plots (Eq. (12)).

The ratio k_{av}/k_{Oli} was determined from the initial rate of AO binding in the absence or in the presence of 0.2 - 0.8 μM oligomycin A at different $[Na^+]$. Table 1 shows representative values of the slopes (i.e., f_1/K_{Oli}) determined from

Table 1. The slope (μM^{-1}, mean ± s.d.) of k_{av}/k_{Oli} vs. [Oli]. The ionic strength, I, was kept constant (upper entries) with TMA. The number of determinations is shown in parenthesis.

$[Na^+]$:	0.15 M	0.96 M	1.43 M
I = 1.9 M	0.85 ± 0.16 (3)	3.47 ± 0.05 (3)	3.34 ± 0.09 (4)
I = $[Na^+]$	0.44 ± 0.09 (3)	-	3.7 (1)

plots of k_{av}/k_{Oli} vs. [Oli] (Eq. 12) as a function of $[Na^+]$. A plateau was reached at 1 M Na^+. If this is taken to indicate that above 1 M Na^+ essentially all the enzyme is E_1P, the slope above 1 M Na^+ = $1/K_{Oli}$, from which we determined K_{Oli} = 0.3 μM. Because K_{Oli} is determined at high ionic strength whereas we are interested in the $[E_1P]$ at physiological ionic strength, we determined the value of the slope at 150 mM Na^+ in the presence and in the absence of 1.8 M TMA. The slope was larger in the presence of TMA. Either TMA has a Na-like effect or K_{Oli} decreases at high ionic strength, or both. The two extremes, obtained with or without TMA, were:

$$\frac{[E_1P]_{TMA=0}}{[E_T]} = 13\% \qquad \frac{[E_1P]_{TMA=1.8\,M}}{[E_T]} = 25\%.$$

The above values are the average of the E_1P fractions in each (Na,K)-ATPase isoform. In contrast to the brain preparation, similar measurements with the dog kidney enzyme (not shown) suggest that the E_1P fraction is about one order of magnitude smaller in this tissue (Lee and Fortes, 1985).

DISCUSSION

Is E_1P an intermediate in the presence of both Na^+ and K^+?
In the brain preparation at 37°C the ratio of (Na,K)-ATPase and Na-ATPase activities is about 30. A value of the fractional E_1P concentration in the absence of K^+ in the range 13 - 25% precludes it from being increased by this factor in the presence of K^+, provided that both (Na,K)-ATPase isoforms have similar kinetic properties. For E_1P to be an intermediate also in the presence of K^+, the reverse rate constant, at 150 mM Na^+, from E_2P to E_1P must be much larger than the dephosphorylation rate constant of E_2P in the absence of K^+. This in turn implies that all the phosphoenzymes can interconvert rapidly in the absence of K^+. If so, all the EP observed in the presence of Na^+ alone should disappear as rapidly upon the addition of K^+ or ADP alone as when both ADP and K^+ are added together. The experiments known to date (e.g. Klodos and Nørby (1987), Yoda and Yoda (1986)) do not appear to conform to this prediction. Models that include E_1P as an intermediate in the presence of K^+ must explain the differences in dephosphorylation patterns observed between addition of ADP or K^+ alone, or both together. Otherwise, a different hydrolysis pathway may be operative in the presence of K^+ and high ATP, as has been proposed (Plesner et al., 1981).

CONCLUSIONS

1. Fluorimetric determinations of AO binding capacity give the same values of active sites as those determined by phosphorylation.
2. AO binding and release kinetics are consistent with two (Na,K)-ATPase isoforms present in roughly equal amounts in ox brain and having similar affinities for AO, with K_D = 15 - 30 nM.
3. The dependence of AO binding kinetics on Na concentration is consistent with at least three phosphorylated interme-

diates in each isoform, as has been proposed by others (Lee and Fortes (1985), Nørby et al. (1983), Klodos and Nørby (1987), Yoda and Yoda (1983)).

4. The concentration of E_1P during steady state turnover with 150 mM Na at 37°C is 13 - 25% of the total brain enzyme. This value is an average of the E_1P of the two isoforms.
5. The steady state levels of E_1P place restrictions on the values of the rate constants for interconversion between phosphoenzymes, if E_1P is an intermediate during turnover in the presence of K. Dephosphorylation experiments appear to be inconsistent with these restrictions.

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AMINE BASE-DEPENDENT INACTIVATION OF Na,K-ATPase: TWO DIFFERENT ASPECTS

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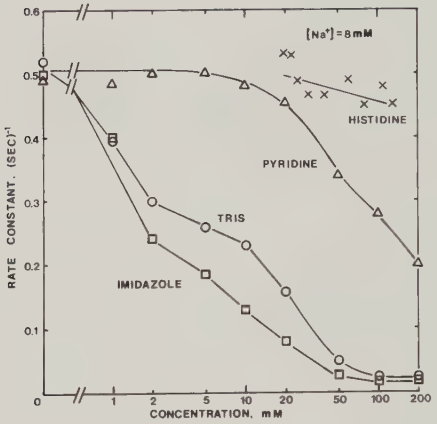
In this report, I will describe two independent experiments on the poisoning action of amine compounds on Na,K-ATPase. They deal with quite different inactivations of this enzyme, but in both cases, protonated amines were toxic while deprotonated form was much less toxic.

1. Inhibition by Amines in the Hydrolysis of Phosphoenzyme

For the extracellular release of sodium bound to a high-affinity site of Na,K-ATPase, a specific aspartate residue of the enzyme must be phosphorylated to reduce the binding affinity for the sodium. This is probably the basis for the utilization of ATP-hydrolysis energy by membrane ATPases to transport ions against concentration gradient. In order to understand the molecular mechanisms of ion-transport by this type of ATPase, I studied the characteristics of the phospho-enzyme of Na,K-ATPase prepared from pig kidney.

The reactivity of the acyl-phosphate of the phospho-enzyme with water was reduced in the presence of amine bases, including usual buffer substances such as Tris or imidazole. The concentration giving half-maximum inhibition was about 2 or 6 mM, respectively (Fig.1). On the other hand, pyridine or histidine was much less inhibitory, although the latter contains the imidazole moiety. Judging from the pKa values of these substances, the protonated amine is probably the inhibitory form. Lysine and arginine, but not glycine, were also relatively strong inhibitors.



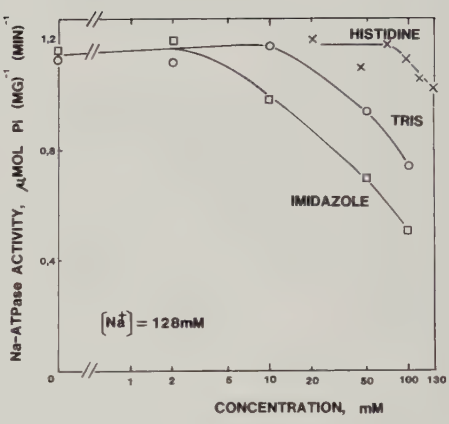


Figure 1. (left) Amine base-dependent inhibition of phosphoenzyme hydrolysis in the presence of 8 mM NaCl, 25 mM histidine, 20 μM radioactive ATP, and 0.5 mM $MgCl_2$ at pH 7.4 and 0°C.

Figure 2. (right) Amine base-dependent inhibition of Na-ATPase in the presence of 128 mM NaCl, 25 mM histidine, 3 mM ATP, and 4 mM $MgCl_2$ at pH 7.4 and 37°C.

Since most published experiments have been performed in the presence of large amounts of Tris or imidazole, the reactivity of the phosphoenzyme with water would have been substantially underestimated. Moreover, this inhibitory effect was also observed in the Na-ATPase without added K (Fig.2).

Sucrase is also known to be inhibited by Tris. This fact might lead one to imagine that amines are general inhibitors of hydrolase reactions. However, there was no inhibition of hydrolysis of the phosphoenzyme of Ca-ATPase in sarcoplasmic reticulum by amines (see Fig.3 of Fukushima, 1987).

By following the dephosphorylation kinetics in histidine buffer without inhibitory amines, Na was also found to be inhibitory (Fig.3). The inhibition reached the maximum level at around 50 mM. This was not seen in the usual reaction mixture, because the inhibition was saturated by Tris or imidazole. Consequently, only the stimulation at higher concentrations of Na has been reported in the literature, since the activation was not affected by amines. Briefly, Na is an activator only at high concentrations under inhibited conditions, but under non-inhibited conditions, Na is an inhibitor at low concentrations (see also Stekhoven et al, 1986).

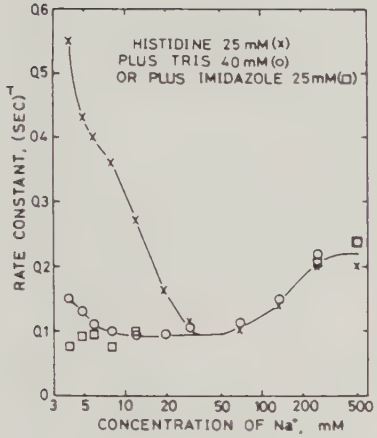
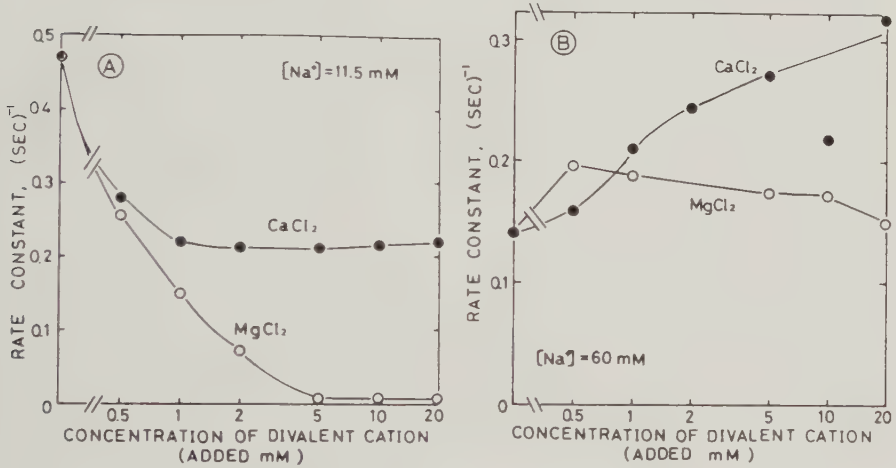


Figure 3. (left) Different effect of Na on the phosphoenzyme hydrolysis in the presence and in the absence of inhibitory amines. See the legend of Figure 1 for the conditions of dephosphorylation.
Figure 4. (under) Inhibition (A)or activation (B) of phosphoenzyme hydrolysis by divalent cations depending upon the concentration of Na. See the legend of Figure 1 for other conditions of dephosphorylation.



Similarly, divalent cations acted as an activator or an inhibitor depending upon the presence or absence of the inhibitory amines or Na. For example, in the presence of an inhibitory amount (60 mM) of Na, Ca was an activator and Mg was also a weak activator (Fig.4A). In the presence of a lower concentration of Na (11.5 mM), Mg was an inhibitor and Ca was a weaker inhibitor (Fig.4B). Further, Ca apparently activated the suppressed dephosphorylation in the presence of imidazole, but Ca inhibited the dephosphorylation in simple histidine buffer (see Fig.9 of Fukushima, 1987).

Amine bases, Na, and divalent cations appear to compete with each other for the same site on the phosphoenzyme, and

it seems likely that divalent cations, especially Ca, were less inhibitory than Na or amines, so that divalent cations apperared to be activators in the presence of other inhibitors.

To explain the mechanism of reduction of the reactivity of the phosphoenzyme with water by amines, Na, or divalent cations, I postulate that these substances bind competitively to a site which contains a water molecule-activating residue (Fig.5). The H_2O is in a specific orientation on the enzyme molecule.

In terms of the Post-Albers scheme, the reactivity of the phosphoenzyme with water greatly increases following the release of bound Na outside the cell. Therefore I further postulate that the water-activating site is a residue after the release of Na. Probably this low-affinity Na site accepts not only Na at high concentration but also divalent cations or amines at millimolar concentrations and consequently loses the ability to activate the H_2O. Similarity between Na and amines in terms of effect on Na,K-ATPase was reported by Stekhoven et al (1985) and Skou & Esman (1983). If this is true, the H_2O-activating Na-releasing site (for example, a carboxyl group in Fig.5) should be near the phosphoryl group that is located in the cytoplasmic part of the enzyme. Actually according to O'Connor & Grisham (1980), the aspartyl-phosphate and Na-binding site(s) may be close enough to interact. Thus, determination of whether Na is inside or outside the cell occurs within the ATPase molecule at the cytoplasmic domain (Fig.6). This mechanism was named "mobile gate model" by Yamamoto et al (1979).

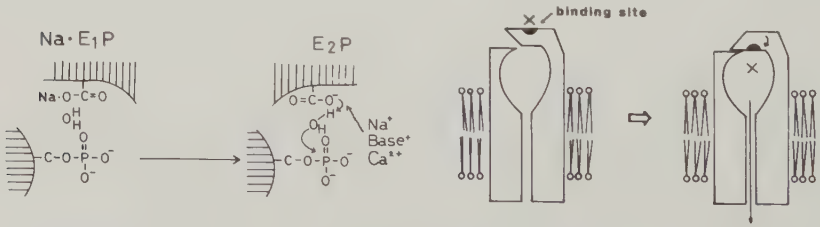


Figure 5. (left) Activation of the water molecule by the low-affinity Na-site, and its inhibition not only by Na but also by amine bases or by divalent cations.
Figure 6. (right) Mobile gate model for the transport of Na, in which the sidedness of Na is determined at the cytoplasmic domain of the Na,K-ATPase.

2. Amine-Dependent Inactivation by Reducing Agent

Amine bases are actually more general inactivators of the Na,K-ATPase than described above. Their inactivating effect was clearly seen by incubating the enzyme with dithiothreitol (DTT) and SDS in the presence of Tris or imidazole (Fig.7). Probably, the structure of the enzyme becomes more fragile as a result of reductive cleavage of disulfide bond(s), and amines can then easily attack the enzyme to inactivate it. About 18 mM Tris or imidazole gave the half-maximum inactivation with DTT. In this case also, histidine was a relatively weak inactivator.

Amine compound did not act as catalysts for DTT reduction, but were themselves inactivators, since the enzyme which had been repeatedly freezed and thawed lost 35 % of its activity with Tris alone without DTT (Fig.8). Most bases could not attack the enzyme in its normal state, but could enhance the inactivation of enzyme that had been partially denatured for some reason or other. Among the tested amines, arginine was the strongest inactivator. At 60 mM (pH 7.4)

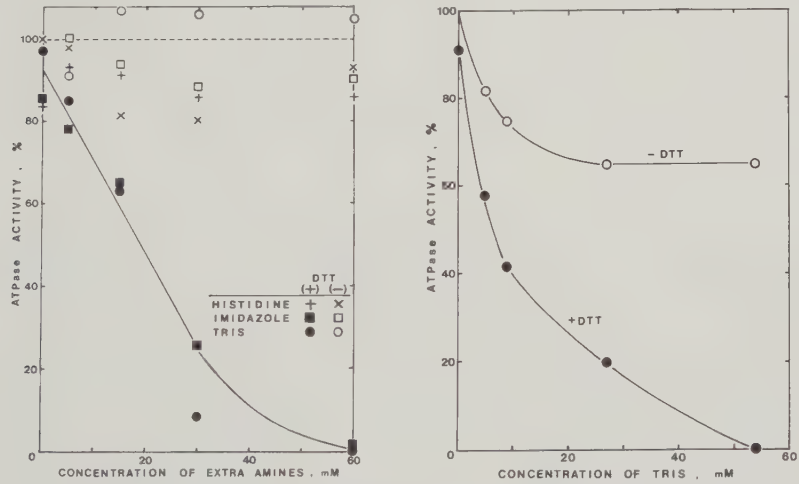


Figure 7. (left) Inactivation of washed Na,K-ATPase after incubation with extra amine bases in the presence of 0.3 M DTT, 0.0786 mg/ml SDS, 0.825 mg/ml membrane proteins and 25 mM histidine (pH 7.4) at 1.5 - 6°C.

Figure 8. (right) Partial inactivation of an aged preparation of Na,K-ATPase washed after incubation with extra Tris in the absence of DTT; other conditions were the same as in the legend of Figure 7.

in the presence of SDS, it completely inactivated even the fresh enzyme without DTT as well as with it, after incubation for 1 h in an ice-bath. However, removel of SDS too from the incubation mixture reduced the extent of inactivation to about 20 %.

In the presence of Na, or K, the enzyme resisted the inactivation by DTT plus amine bases. The monovalent cation concentration for protection was dependent on the concentration of amines. For example, 10 mM or 0.1 mM KCl maintained about 90 % activity in the presence of 15 or 30 mM imidazole, respectively. On the other hand, ATP was not an effective stabilizer. The inactivating amines may bind to the monovalent cation-binding site (probably an acidic residue) preferentially, but not to the ATP site.

The requirement of SDS or SDS plus DTT for inactivation by amines means that the target site(s) of these inhibitors is in the hydrophobic region of the Na,K-ATPase. If this is the monovalent cation-binding site as argued in the above paragraph, the target site(s) of the inhibitor may exist in or near the channel structure (occlusion form) involved in ion transport.

I am indebted to Akiko Miwa for technical assistance and to Keiko Fujiwara for preparing the enzyme.

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COMPLEX TIME DEPENDENCE OF PHOSPHOENZYME FORMATION AND DECOMPOSITION IN ELECTROPLAX Na,K-ATPase

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INTRODUCTION

Considerable evidence supports the Na,K-ATPase mechanism proposed independently by Albers and Post and their collaborators a number of years ago, which with subsequent modifications has continued to explain most of the observations made in the intervening years. The model contains two major phosphorylated and two major dephosphorylated conformations of the enzyme (E_1-P,E_2-P, E_1,and E_2), which are experimentally distinguished by their affinities for, and reactivities to, various activating and inhibiting ligands.

However, evidence obtained in the last few years suggests that further, more radical modifications may be necessary to account for complex patterns of enzyme behavior which do not appear to be explained by this model. Several lines of evidence suggest the simultaneous existence of more than one nucleotide binding site (Ottolenghi and Jensen, 1983; Askari and Huang, 1984), and multiphasic patterns of phosphoenzyme formation (Hobbs *et al.*, 1983b, Froehlich *et al.*, 1985) and decomposition (Norby *et al.*, 1983, Hobbs *et al*, 1985, Yoda and Yoda, 1986). The subject of this study is the effect of Mg^{2+} on the biphasic pattern of phosphorylation that appears when enzyme is incubated with ATP prior to initiation of phosphorylation and on the multiphasic dephosphorylation that occurs when enzyme phosphorylated in the presence of Na^+ and Mg^{2+} is combined with K^+ and EDTA.

METHODS

Experiments were performed using a quenched-flow device that combines enzyme and substrate in one or two stage mixing operations prior to quenching with perchloric acid (Froehlich *et al.*, 1976). In several experiments, two such devices were coupled in order to do three stage mixing operations. Membrane bound Na,K-ATPase was prepared from electroplax of *Electrophorus electricus,* suspended at approximately 1 mg/ml in the appropriate experimental medium, and mixed with substrate solutions for various times as indicated in individual experiments.

RESULTS

When *Electrophorus* Na,K-ATPase is phosphorylated by the addition of 10 μM ATP, either subsequent to or simultaneously with, Na^+ and Mg^{2+}, the time course of phosphorylation closely resembles a single exponential which follows a short lag (Hobbs *et al.*, 1983b). This behavior has been successfully simulated by assuming a single component of enzyme which is initially present as E_1, and must subsequently bind ATP prior to the phosphorylation step. If this model is correct, allowing ATP to bind first should uncover a more rapid rate, that of the single step $E{\cdot}ATP \rightarrow E_1{-}P$. However, when this experiment is performed (Fig. 1), at least two phases of phosphorylation are observed. Data reported previously by Mardh and Post (1977) also show this biphasic behavior. Fig. 1 also shows that the proportion of these rapidly and slowly phosphorylating components varies with added Mg^{2+}. In 25 μM Mg^{2+} the rapid component is nearly absent, whereas in 200 μM Mg^{2+} it consists of 59% of the total phosphoenzyme. A number of similar experiments, at varying Mg^{2+} concentrations, are summarized in Table 1. It can be seen that the effect of Mg^{2+} is close to saturation at 200 μM, and that while the proportion of rapidly and slowly phosphorylating fractions varies, the rates do not appear to be changing significantly. The total amount of phosphorylation does not change appreciably. These results cannot be explained by the Albers-Post mechanism using any reasonable choice of rate constants. Using the model

$$E_1 + ATP \rightarrow E_1{\cdot}ATP \rightarrow E_1{-}P + ADP \rightarrow E_2{-}P \rightarrow E_2 + P_i$$

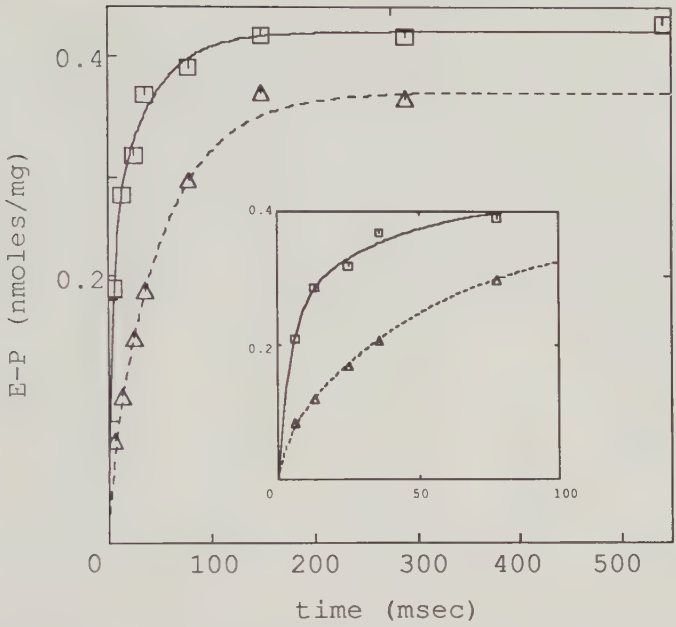

Fig. 1. Biphasic phosphorylation after preincubation with ATP. Enzyme was mixed with 10 mM [γ-^{32}P]ATP for 116 msec prior to the addition of 50 mM NaCl and 25 μM (triangles) or 200 μM (squares) $MgCl_2$ to initiate phosphorylation. Lines are drawn from the equation $E_t=E_f(1-e^{-k_f t})$ + $E_s(1-e^{-k_s t})$ where for 25 μM $MgCl_2$, E_f=.053 nmol/mg, k_f=300 sec^{-1}, E_s=.323 nmol/mg, k_s=18.4 sec^{-1} and for 200 μM $MgCl_2$, E_f=.246 nmol/mg, k_f=229 sec^{-1}, E_s=.174 nmol/mg, k_s=26.5 sec^{-1}. Inset shows an expanded view of the origin.

and previously measured rate constants, simulated E-P formation closely resembles a single exponential at both 10 and 100 μM ATP (Fig. 2). Even though E·ATP is incompletely formed at the lower ATP concentration, a single exponential with a slower rate (122 sec^{-1} *vs.* 184 sec^{-1}) is still able to fit the simulated data quite well.

If formation of E_1-P occurs rapidly and reversibly, and E_2-P forms more slowly, this could produce

TABLE 1

Effect of $[Mg^{2+}]$ on the fast and slow phases of phosphoenzyme formation. Enzyme suspended in Tris HCl, pH 7.45, was preincubated with 10 μM ATP for 116 msec, then allowed to phosphorylate after addition of $MgCl_2$ and 50 mM NaCl. The rapidly (EP_f) and slowly (EP_s) phosphorylating components are expressed as fractions of the total phosphoenzyme (EP_t) and the rates (in sec^{-1}) as k_f and k_s.

Conditions		$[EP_f]/[EP_t]$	k_f	$[EP_s]/[EP_t]$	k_s
$[Mg^{2+}]$, μM	25	.13	>100	.87	19
	50	.36	249	.64	20
	200	.60	220	.40	25
	3000	.65	361	.35	28

a biphasic pattern of phosphoenzyme formation. However, several facts mitigate against this:

1) K^+-sensitive phosphoenzyme (E_2-P) forms too rapidly (>300 sec^{-1}) to account for the slow phase (Hobbs *et al.*, 1985).

2) Almost complete ADP sensitivity is acquired by the oligomycin-treated enzyme at very short phosphorylation times (Hobbs *et al.*, 1983a), suggesting that ADP is released rapidly and quantitatively. This would prevent reversal of E_1-P formation under our experimental conditions. Further addition of phosphorylated intermediates downstream from E_1-P, irrespective of their rates of formation, will not produce a biphasic phosphorylation, as long as the formation of E_1-P is not reversible and E-P breakdown is not significant.

We previously obtained results in dephosphorylation experiments which showed three components of E-P could be measured when K^+ and EDTA were added to enzyme which had been phosphorylated in the presence of Na^+ and Mg^{2+} (Hobbs *et al.*, 1985). The slowest component is explained mainly by inside-out vesicles which are only slowly permeable to K^+; however, both the rapid and intermediate components ought to be able to be ex-

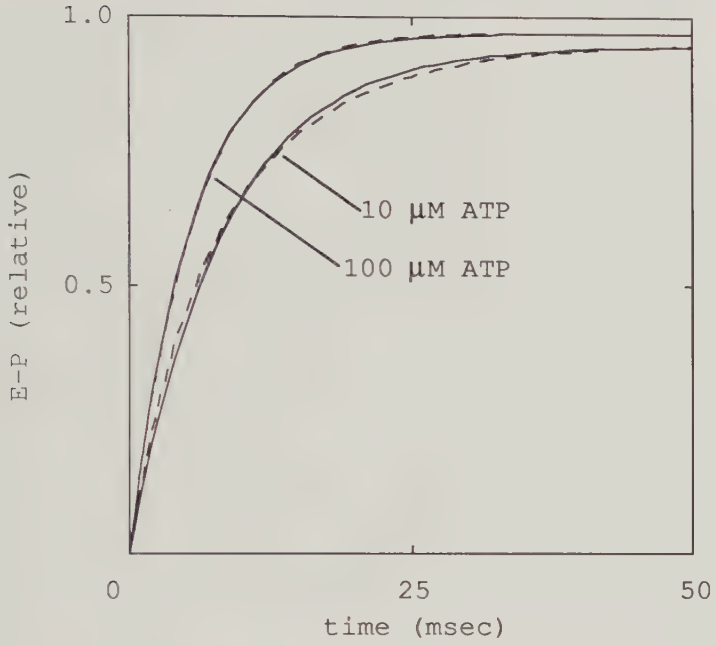

Fig. 2. Theoretical phosphorylation patterns after preincubation of enzyme with 10 or 100 μM ATP. The assumed model in this figure is

$$E_1 \xrightarrow{(k_1)} E_1 \cdot ATP \xrightarrow{(k_2)} E_1\text{-}P \xrightarrow{(k_3)} E_2\text{-}P \xrightarrow{(k_4)} E_2 \xrightarrow{(k_5)} E_1$$

with the following set of rate constants (Froehlich *et al.*, 1976, Hobbs *et al.*, 1985): $k_1=1.2 \times 10^7\ M^{-1}\ sec^{-1}$, $k_{-1}=40\ sec^{-1}$, $k_2=180\ sec^{-1}$, $k_{-2}=0$, $k_3=500\ sec^{-1}$, $k_{-3}=0$, $k_4=2\ sec^{-1}$, $k_{-4}=0$, $k_5=100\ sec^{-1}$, $k_{-5}=0$. In the 10 μM case, initial $E_1=.25$, initial $E\cdot ATP=.75$ and the fitted rate (solid line) is $122\ sec^{-1}$. In the 100 μM case, initial $E_1=.03$, initial $E\cdot ATP=.97$ and the fitted rate (solid line) is $184\ sec^{-1}$. In both cases the simulated data is represented by the dashed line.

plained in the context of the overall pathway of Na-dependent ATP hydrolysis. Our previous analysis of dephosphorylation patterns obtained after different phosphorylation times could not be explained by a scheme in which the fast and slow components of dephosphorylation represent consecutively formed phos-

phoenzyme intermediates as proposed by Norby *et al.* (1983) and Yoda and Yoda (1986). It is interesting to note, however, that the rapid and slow components of phosphorylation at saturating [Mg^{2+}] appear in about the same ratio (2:1) as the fast and intermediate components of dephosphorylation. To test the hypothesis that the rapidly and slowly phosphorylating components might be linked to these two components of dephosphorylation, we performed a number of experiments preincubating the enzyme with ATP, phosphorylating in the presence of low or high Mg^{2+}, and allowing the enzyme to dephosphorylate in the presence of K^+ and EDTA. The underlying assumption is that if the components of the phosphorylation and the dephosphorylation reactions are directly related, varying the Mg^{2+} concentration, which affects the pattern of phosphorylation, should also affect the pattern of dephosphorylation. The results, in Table 2, show no apparent effect of Mg^{2+} on the dephosphorylation under these conditions, suggesting that there is no simple relationship between the components.

DISCUSSION

The patterns of phosphorylation and dephosphorylation shown here do not appear to be compatible with a simple, monomeric enzyme scheme. Two alternative possibilities are the existence of multiple forms of enzyme (isozymes such as αI and αII) or subunit interactions in an oligomeric enzyme. One problem in formulating a model for the behavior shown in this study is that the ratios of the rapidly and slowly phosphorylating and dephosphorylating forms are approximately 2:1 in each case. This means that if oligomeric behavior is involved, it is almost certainly of a higher order than dimerization. While a model of this type might explain the dephosphorylation results, (e.g. two subunits might be rapidly dephosphorylated and the third only slowly under these conditions), the components of dephosphorylation do not appear to be sensitive to changing Mg^{2+} level, while the phosphorylation results are affected by Mg^{2+} in a continuous way. This suggests that the E-P formed slowly, at low Mg^{2+} levels, is the same as, or is converted rapidly to, the E-P formed rapidly at higher Mg^{2+} levels, once phosphorylation has occurred.

Table 2

Dephosphorylation by K^+ and EDTA of E-P formed following ATP preincubation. Enzyme suspended in 60 mM Tris HCl, pH 7.45, was preincubated with ATP for the indicated times, then allowed to phosphorylate after the addition of 25 mM NaCl and the indicated [Mg^{2+}]. After phosphorylation had proceeded for the times indicated, 6.67 mM K^+ and 10 mM EDTA were added and the time course of disappearance of the phosphoenzyme followed. EP_f, EP_i and EP_s represent the fraction of phosphoenzyme disappearing at rapid (200-300 sec^{-1}), intermediate (15-30 sec^{-1}) and slow (0-2 sec^{-1}) rates.

[ATP] (μM)	Preincubation Time	[Mg^{2+}] (μM)	Phosphorylation Time	EP_f (relative)	EP_i	EP_s
15	116 msec	25	145 msec	.54	.26	.21
15	116 msec	1000	145 msec	.55	.10	.35
100	116 msec	1000	145 msec	.61	.18	.21
100	1 min.	50	10 msec	.62	.14	.25
100	1 min.	1000	116 msec	.66	.13	.22
100	1 min.	25	116 msec	.68	.10	.23

Postulating that the biphasic phosphorylation behavior is due to isozymes produces other difficulties. It would appear to require that one enzyme form have a sensitivity to Mg^{2+} levels (the form that becomes rapidly phosphorylating as Mg is raised) that the other form lacks. When the Mg^{2+} site which confers this sensitivity is unoccupied both forms of the enzyme are identical with respect to their phosphorylation and dephosphorylation behavior. Complexation with Mg must alter the kinetics of phosphorylation in one form (converting it from slowly to rapidly phosphorylating), while leaving dephosphorylation unchanged. Although such behavior may occur, it places severe constraints in formulating a mechanism for the system. Thus, while the Albers-Post model appears to be inadequate in accounting for the behavior described here, none of the alternatives

seems entirely satisfactory, either. Future efforts will have to be directed toward finding ways to test these and perhaps toward finding new alternatives.

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ATP SYNTHESIS BY Na,K-ATPase. EFFECT OF K^+ ON THE ADP-DEPENDENT DEPHOSPHORYLATION OF PHOSPHOENZYMES.

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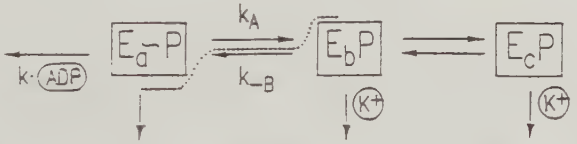
INTRODUCTION

During Na-ATPase activity of the Na,K-pump the enzyme forms acid-stable phosphointermediates, EP, and it should now be generally accepted that at least three distinct EP-pools must be part of the reaction mechanism (Glynn, this volume; Nørby and Klodos, this volume). In recent papers we have described this part of the reaction cycle by using a consecutive three-pool model:



and we investigated the properties of this model by studying dephosphorylation kinetics in the presence of ADP or K^+ (Nørby et al., 1983; Klodos and Nørby, 1987). E_a~P and E_bP, provided that k_{-B} is relatively large, will be dephosphorylated by ADP, resulting in resynthesis of ATP.

E_bP and E_cP are both rapidly hydrolyzed to E and P_i in the presence of K^+. The dephosphorylation curves are biphasic (in a semilog plot) and according to the model the slow phase, $k = 0.1\ s^{-1}$ with ox brain enzyme at 0°C, should reflect the transition of E_a~P to E_bP plus whatever spontaneous dephosphorylation of E_a~P there may be. This result of K-dephosphorylation experiments is out of accordance with previously measured rates of the interconversion between EP's (Post et al., 1969; Hobbs et al., 1985, Klodos, unpublished observations). We have therefore postulated that K^+ blocks the conversion of E_a~P to E_bP (Nørby et al., 1983).

One way to test the hypothesis of K^+-blockage of the E_a~P ⟶ E_bP conversion is explored in this communication by studying ATP synthesis from E_a~P under conditions where k_A

and k_{-B} are varied - by changing the Na^+-concentration - in the presence and absence of K^+, i.e. in the presence or absence of (E_bP+ E_cP). ATP synthesis was probed with μM ADP to allow some E_a~P to "escape" to E_bP. If the sole action of K^+ is to remove the K-sensitive EP-forms, then the ATP synthesis observed in the presence of K^+ would be always lower than in its absence, since with K^+ the rate $k_{-B} \cdot E_bP$ is effectively 0. If, however, K^+ also blocks E_a~P to E_bP then conditions might be found where, with a very low k_{-B}, blockage would result in a higher ATP-synthesis. The argument is illustrated here by the three-pool model, but any number of consecutive EP pools (greater than 1) could be used as well.

The results obtained here with Na^+ < 20-40 mM suggest that the action of K^+ indeed goes beyond the rapid removal of K-sensitive EP-forms and support the hypothesis that K^+, when added to dephosphorylate the EP formed in the absence of K^+, blocks the transition of K-insensitive to K-sensitive forms.

MATERIALS AND METHODS

Na,K-ATPase was prepared from ox brain as described by Klodos et al. (1975), and from pig kidney by the SDS procedure of Jørgensen (1974) with the modifications given by Jensen et al. (1984). The Na,K-ATPase activities (in U·(mg protein)$^{-1}$) and the phosphorylation sites (in pmol·(mg protein)$^{-1}$) were: ~ 3.5 and 380 for the brain and ~ 12 and 1600 for the kidney enzyme.

The enzymes were phosphorylated in the presence of 200 or 400 μM ATP, 0.1 mM $MgCl_2$, 30 mM imidazole buffer, pH 7.4 and 1 to 600 mM NaCl. Phosphorylation time was 10 seconds at 0°C and 2 seconds at 20°C. Phosphorylation was stopped by the addition of 10 mM EDTA. Simultaneously with the chelator ^{3}H-ADP was added without or with 10 mM KCl. The following incubation period was 1-5 minutes at 0°C or 30-90 seconds at 20°C. The reaction was stopped by the addition of 5% TCA and samples were centrifuged in a Beckman Airfuge. The protein-free supernatants were neutralized and the amount of ATP formed was measured after separation of nucleotides by thin-layer chromatography (Cashel et al., 1969). In control experiments it was shown that 100% of the added radioactivity was found in the ATP, ADP and AMP spots.

All experiments described in the present report were done in the presence of 3 μM ^{3}H-ADP. There was no measurable adenylate kinase activity in either enzyme preparation in the experimental conditions used. The amount of ATP formed

was calculated after subtraction of the radioactivity found in ^{3}H-ATP spots in enzyme free blanks.

The "ATP-burst", i.e. the amount of ATP formed "instantaneously" from E_a~P, was determined by extrapolation of the time course of the ADP-ATP exchange reaction - which still proceeds even with 10 mM EDTA and only 0.1 mM $MgCl_2$ - to time zero (shown in Fig. 1). All reagents used were reagent grade. ATP and ^{3}H-ADP were purified and converted to their $Tris^+$ salts by chromatography as described earlier (Nørby and Jensen, 1971).

RESULTS

Oligomycin blocks the transition between the ADP-sensitive and the K-sensitive EP (Post et al., 1969), and therefore one would expect that all the phosphoenzyme formed in the presence of this inhibitor would react with ADP giving rise to ATP formation. The oligomycin-blocked enzyme was hence used to evaluate the validity of the applied method. These control experiments, shown in Figure 1, reveal that ADP, even as low as 3 μM, reacts effectively and quantitatively with E_a~P to synthesize ATP. The conversion - "ATP burst" - is rapid in comparison with the rate of ADP-ATP exchange and the total amount of ATP formed in the "ATP burst" is equal to the amount of EP in the experiment. Moreover, the experiment shows that the amount of ATP formed as well as the exchange rates are independent of the presence of K^+ during the reaction with ADP.

It was previously shown that the amount of ADP-sensitive EP increases with a rise in Na^+ concentration and the results of experiments performed with brain enzyme at 0°C, in which the ATP synthesis with and without K^+ was measured as a function of Na^+, are shown in Figure 2.

The amount of ATP formed, both with and without K^+, increases with an increase in Na^+ concentration, as one could expect. But, whereas K^+ added together with ADP decreases the ATP synthesis when Na^+ is higher than 20 mM, then at Na^+ concentrations below 20 mM there is more ATP formed in the presence of K^+ than in its absence.

From the arguments that follow below it is rendered probable that the previously hypothesized blocking of the E_a~P—> E_bP conversion could explain the difference in effect of K^+ at low and high Na^+. The argument requires consideration of the rate constants and the fractional distribution of EP-species as determined earlier (see scheme in Introduction; Nørby et al., 1983) and shown in Table 1.

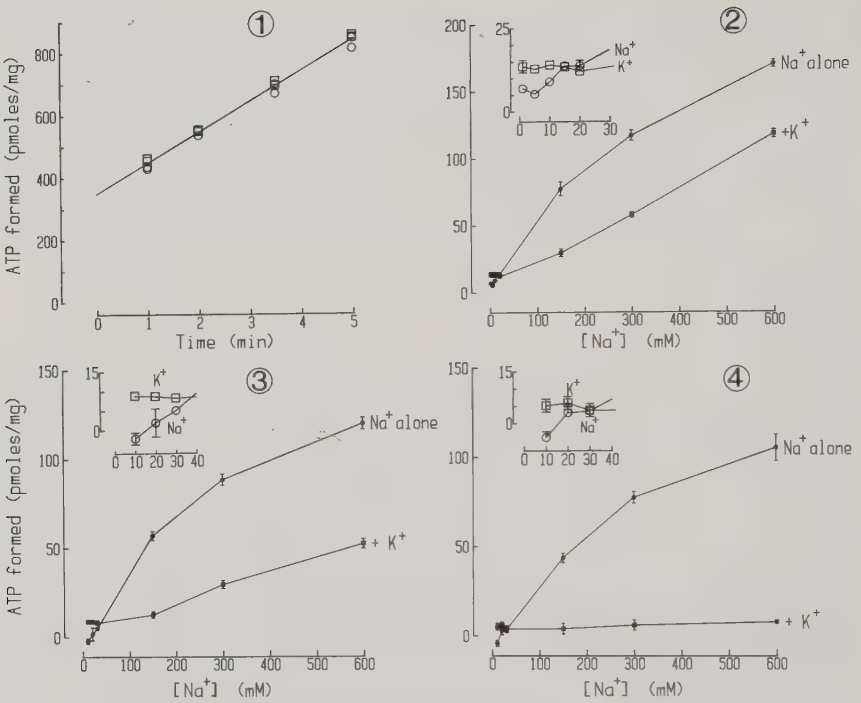


Figure 1. ADP-ATP exchange and "ATP burst" with oligomycin-treated brain enzyme phosphorylated in the presence of 400 μM ATP, 0.1 mM $MgCl_2$, 300 mM Na^+ and 30 mM imidazole buffer, pH 7.4 at 0°C, and 20 μg oligomycin ml^{-1} (50 μg (mg protein^{-1})). Exchange reaction was measured after addition of 10 mM EDTA, 3 μM ^{3}H-ADP and (o) no K^+, or (□) 10 mM K^+.

Figure 2. "ATP burst" as a function of Na^+. The experiments were carried out as in Figure 1 omitting oligomycin. (o) no K^+, (□) 10 mM K^+. The results are average ± SEM of at least 3 experiments.

Figure 3. "ATP burst" as a function of Na^+ with pig kidney enzyme at 0°C. The experiments were carried out in the presence of 200 μM ATP. All other experimental conditions as in Figure 1 omitting oligomycin. (o) no K^+, (□) 10 mM K^+.

Figure 4. "ATP burst" as a function of Na^+ at 20°C. Experiments with pig kidney enzyme as in Figure 3.

Table 1. E_a~P, E_bP and E_cP and transition rate constants k_A and k_{-B} as functions of Na^+ for the brain enzyme at 0°C. EP concentrations in % and rate constants in s^{-1}, see scheme in Introduction. Data from Nørby et al. (1983).

Na	E_a~P	E_bP	E_cP	k_A	k_{-B}
20	12	45	43	0.65	0.09
150	19	43	38	1.20	0.43
300	32	40	28	1.41	0.95
600	51	34	15	1.21	1.80

At low Na^+ the "ATP burst" is <u>increased by</u> K^+ (Fig. 2). It may be seen from Table 1, that at low Na^+ (e.g. 20 mM) the "back rate constant" for the transition, k_{-B}, is small compared to the "forward constant", k_A. Here removal of E_bP, if this was the only K^+ effect, should be of minor consequence for the size of the "ATP burst", but if K^+ were to block simultaneously E_a~P —> E_bP then the "ATP burst" could be increased by K^+ as observed.

At high Na^+ the "ATP burst" is <u>decreased by</u> K^+ (Fig. 2). This is also explained by the rate constants in Table 1. At high Na^+ there will be a net conversion of E_bP to E_a~P after ADP addition and E_bP will thus contribute to the "ATP burst". This contribution is of course eliminated by K^+, which removes E_bP and hence reduces the "ATP burst".

The ratio of ADP- to K-sensitive phosphoenzymes depends not only on Na^+ but also on the source of enzyme. It was previously shown (Klodos and Nørby, 1987) that the pig kidney enzyme at 150 mM Na^+ reacts similarly to the brain enzyme at 20 mM Na^+ with respect to effects of Na^+ on the properties of EP's. It would therefore be expected that with the enzyme from kidney the inhibitory effect of K^+ on the "ATP burst" should appear at higher Na^+ than with the brain enzyme, and this is indeed the case (Figures 3 and 4). Experiments in Figure 3 were performed at 0°C and those in Figure 4 at 20°C, showing that the conclusions based on dephosphorylation experiments done at 0°C seem to be valid also at higher temperatures.

<u>In conclusion</u>: The results obtained support our hypothesis (Nørby et al., 1983) that K^+ blocks the conversion of ADP-sensitive EP to K-sensitive EP, when added to phosphoenzymes formed in the presence of Na^+ alone. This seems to be the case at 0°C with both ox brain and kidney enzyme and 20°C with kidney enzyme.

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DOES ATP AFFECT THE INTERCONVERSION AND THE DEPHOSPHORYLATION OF THE PHOSPHOENZYMES OF THE Na,K-PUMP?

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INTRODUCTION

The purpose of the present communication is to elucidate any effect of ATP on those phosphointermediates (EP) that are formed in the presence of Na^+ alone. These EP's are thought to be intermediates of the Na-ATPase reaction - and various Na-transport modes - and by many also to be intermediates in the Na,K-ATPase reactions.

There are several reasons why it is important to establish whether ATP, especially in the concentration range 10 - 1000 μM, has any modulating effect on the part of the reaction cycle represented by the EP's. Here we mention three:

1. It has been shown for Ca-ATPase from the sarcoplasmic reticulum (see Andersen and Vilsen, this volume) that ATP can increase the rate of hydrolysis by increasing the rate a) of $E_1{\sim}P \longrightarrow E_2P$, b) of hydrolysis of E_2P or c) of conversion of E_2 to E_1, depending on the conditions. For Na,K-ATPase, so far only the last possibility for millimolar ATP modulation has been demonstrated.

2. ATP interaction with the EP's might disclose an ATP site or an ATP subsite on the phosphorylated peptide.

3. The interpretation of phosphorylation - dephosphorylation kinetics is often based on comparison of the steady-state phosphorylation at one $AT^{32}P$ concentration (usually in the μM-range), with the dephosphorylation time course of $E^{32}P$ after addition of a much larger (mM) concentration of unlabelled ATP to quench further $E^{32}P$ formation.

The question of ATP interaction with EP from Na,K-ATPase has previously been directly adressed by two groups both using Na,K-ATPase from dog kidney. Askari and Huang (1982) studied EP formed with Mg^{2+} and inorganic phosphate (in the absence of Na^+). This EP was insensitive to both K^+ and ADP (and thus not likely to be an intermediate in the

Na,K-ATPase reaction) and its dephosphorylation was inhibited by both ATP and ADP. This inhibition disappeared upon addition of Na^+. Fukushima et al. (1984) phosphorylated the enzyme with 50 μM ATP, 50 μM $MgCl_2$ and 100 mM Na^+. The EP produced was insensitive to ADP but sensitive to K^+. Also here ATP (and ADP, AMP and various other ATP analogues) inhibited the spontaneous dephosphorylation (in the absence of K^+) but only at very low Mg^{2+} concentration. There was no effect in the presence of K^+. These results might illustrate that binding of ATP to at least some types of EP can take place. However, since the effect of mM concentrations is to accelerate hydrolysis in the Na,K-ATPase reaction and since it is absent in the Na-ATPase reaction (Glynn and Karlish, 1976), the significance of these observations is not clear.

In the following we report on an investigation of the possible effect of mM concentrations of ATP on EP under conditions where both Na^+, K^+ and Mg^{2+} are present. From the results we conclude that the ATP effect, if present at all, is limited to a marginal inhibition of the dephosphorylation of E_2P in the absence of K^+.

MATERIALS AND METHODS

The ox brain Na,K-ATPase (Klodos et al., 1975) had the same properties as that used in our previous investigations (Nørby et al., 1983; Klodos and Nørby 1987): specific activity ~ 4.5 U·(mg protein)$^{-1}$, phosphorylation sites ~ 500 pmol·(mg protein)$^{-1}$. Phosphorylation of the enzyme was accomplished at 0°C in 60 sec. with 1 mg enzyme·ml^{-1}, 0.1 mM $MgCl_2$, 30 mM imidazole buffer pH 7.4 and the Na^+- and [γ-^{32}P]-ATP concentrations shown in the Figures. $E^{32}P$ was determined after acid precipitation as described earlier (Nørby et al., 1983). All reagents used were reagent grade. ATP and [γ-^{32}P]-ATP were purified and converted to their $Tris^+$ salts by chromatography as described by Nørby and Jensen (1971).

RESULTS

ATP and the K-sensitive EP

As mentioned above, Fukushima et al. (1984) demonstrated an ATP inhibition of the spontaneous dephosphorylation of E_2P from kidney (at low Mg^{2+} and no K^+). Here we investigated this phenomenon with the ox-brain enzyme, phosphorylated with 25 μM ATP in the presence of 20 mM, 150 mM, or 300 mM Na^+ and also with 20 mM Na^+ + 280 mM $Tris^+$.

According to our previous analysis the K-sensitive EP should prevail at both 20 and 150 mM Na^+, whereas at 300 mM Na^+ and at 20 mM Na^+ + 280 mM $Tris^+$ a substantial part of EP should be in the K-insensitive form (Nørby et al., 1983; Klodos and Nørby, 1987).

Phosphorylation by $AT^{32}P$ was stopped and dephosphorylation of $E^{32}P$ initiated by 1) addition of 1 mM unlabelled ATP, 2) removal of Mg^{2+} by 10 mM EDTA or 3) addition of both 1 mM unlabelled ATP and 10 mM EDTA. In parallel experiments 10 mM K^+ was added simultaneously with the chase.

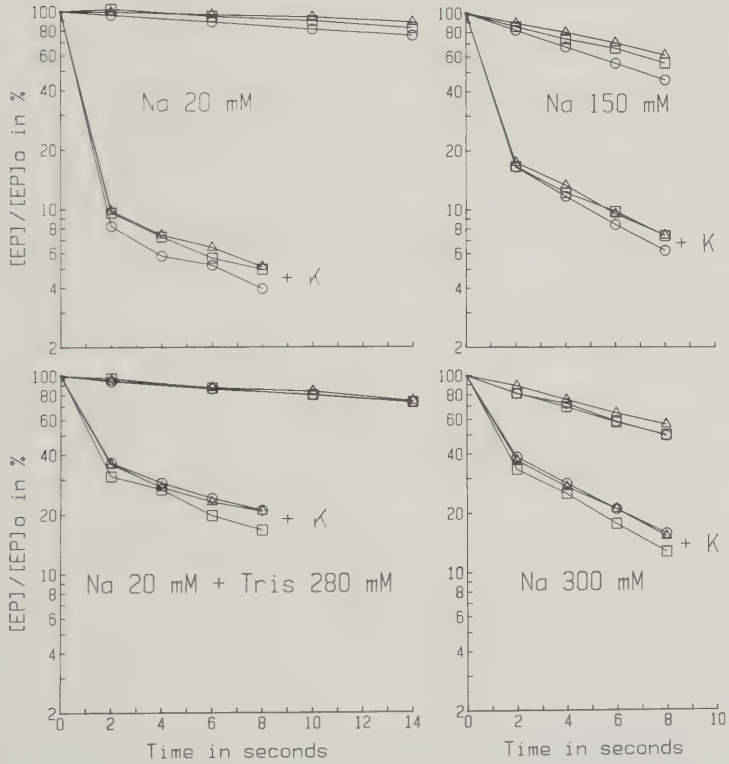

Figure 1. Dephosphorylation of $E^{32}P$ in the presence of (□) ATP 1 mM, (o) EDTA 10 mM, or (△) EDTA 10 mM + ATP 1 mM with or without 10 mM K^+, added at time 0. Phosphorylation was with 25 μM $AT^{32}P$ and Na^+ and $Tris^+$ as indicated. This medium was only diluted 3% by the chase.

Figure 1 shows that ATP barely affects the rate of spontaneous dephosphorylation and only so, when K-sensitive EP constitutes a large proportion – "Na 20mM" and "Na 150 mM". The dephosphorylation rate and the proportion of K-insensitive EP is not influenced by ATP.

The marginal effect of ATP seen in Figure 1 could be due to a saturation of the ATP effect already at 25 μM ATP. The results in Figure 2 do not support this notion . Increasing ATP from 0.8 μM (which is actually only about 5 times K_D for the high affinity site) to 3 mM is virtually without further effect on the dephosphorylation curves.

ATP and the Interconversion of EP-species

The last problem adressed was: will ATP influence the interconversion of EP forms like it does for Ca-ATPase, where $E_1P \rightarrow E_2P$ is accelerated by mM ATP?

In these experiments (Figure 3) the proportion of E_1P in steady-state was varied by phosphorylating the enzyme with 25 μM $AT^{32}P$ and 20, 150 or 300 mM Na^+. The proportion of K-sensitive EP was then determined in steady-state or after a 4-6 seconds of "rearrangement" period following the addition of the chase solution.

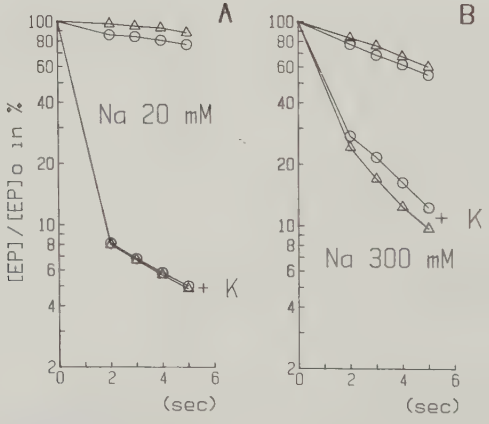

Figure 2. Dephosphorylation of $E^{32}P$ in the presence of (o) 0.8 μM $AT^{32}P$, 10 mM EDTA, 0.1 $MgCl_2$; (Δ) 0.8 μM $AT^{32}P$, 3 mM ATP, 10 mM EDTA, 0.1 mM $MgCl_2$. The enzyme was phosphorylated with 5 μM $AT^{32}P$ and Na^+ in the concentrations shown. At zero time the assays were diluted with assay medium to give the composition indicated above (Na^+ and buffer concentration was not changed).

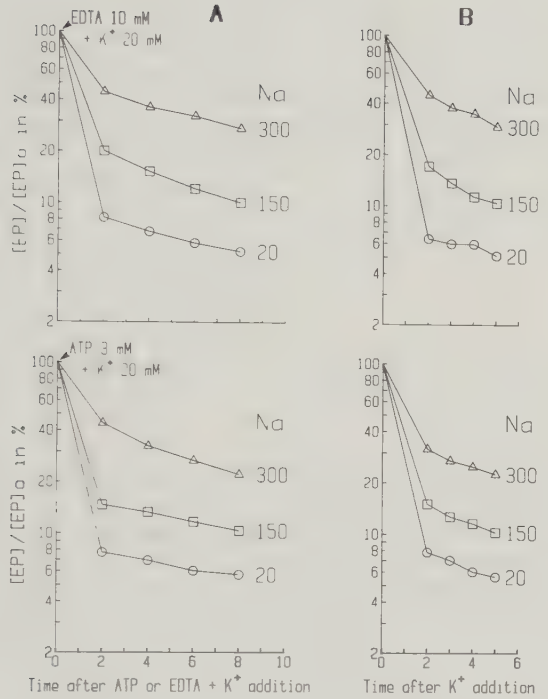

Figure 3. Lack of effect of ATP on the proportion of K-sensitive EP. K^+ was added with (panel A) or 4-6 seconds after the chase (panel B). Time 0 is the time of K^+ addition.

The K-dephosphorylation curves after EDTA or ATP chase are practically identical (Figure 3 upper and lower panel). Comparison of panel A, that illustrates the steady-state ratio of K-sensitive to K-insensitive EP, with panel B, showing the same ratio after 6 sec. (20 mM Na^+) or 4 sec. of "rearrangement", reveals that: 1) ATP neither influences the interconversion of the phosphointermediates nor their proportion and 2) the transition between EP-forms must be rather fast in comparison with the spontaneous dephosphorylation, i.e. the EP's behave as an equilibrium mixture.

The conclusion of these experiments is, that in the presence of Na^+ and Mg^{2+} there is only a marginal, if any, effect of ATP on the properties of the EP's of Na,K-ATPase.

Likewise dephosphorylation kinetics in the presence of K^+ is unaffected by ATP in the range 0.8 μM - 3 mM. This is in agreement with previous results (e.g. Taniguchi et al., 1986; Klodos and Nørby, 1987 p. 309, 311). For further discussion of the possible interaction of the EP's with nucleotides see Askari and Nørby and Klodos, this volume.

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INTERACTION OF THE Na,K-ATPase WITH H_2DIDS

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INTRODUCTION

Chemical modification of the Na,K-ATPase has been used to identify specific amino acid residues which are essential for the enzyme activity. Thus, the amino acid that is phosphorylated is asp-376 (in T. californica enzyme) and FITC (fluoresceine isothiocyanate) binds to lys-507 (Kawakami et al., 1985).

Even though the amino acid sequence of the α and β subunits of the Na,K-ATPase have been described (Shull et al., 1985; Kawakami et al., 1985; Schneider et al., 1985) most of the important structural features of the enzyme are still unknown. These involve the spatial relationships between different regions of primary structure, the location within the primary structure of functionally important amino acids and the way each of the subunits of the enzyme interact and are folded through the cell membrane. Thus, covalent modifications with a variety of reagents followed by the study of the fragments produced by treatment of the modified protein with proteolytic enzymes like trypsin should provide important information relating structural and mechanistic properties of the enzyme.

In this communication, we examine the covalent interaction of dihydro 4,4'-diisothiocyano stilbene 2,2'-disulfonate (H_2DIDS) with the Na,K-ATPase and the subsequent inhibition of the enzyme activity.

METHODS

The enzyme was purified according to Liang and Winter (1976). Following purification the enzyme was washed free of glycerol with 25 mM imidazole, 1 mM EDTA, pH (20°C) 7.5. Na,K-ATPase and pNPPase activities were measured as previously described (Pedemonte and Kaplan, 1986). All inhibitors were dissolved in water. Phosphorylation was measured following Askari and Huang (1982) and ADP binding according to Robinson (1980). Electrophoresis was made following Laemmli (1970) on a linear gradient gel (8-14%). Trypsin treatmment was performed as reported by Jorgensen (1975).

RESULTS

Fig. 1 shows the reversible inhibition of Na,K-ATPase and pNPPase activities by H_2DIDS. This kind of inhibition was observed when the interaction of H_2DIDS with the enzyme was examined in the presence of the enzyme ligands. Kinetic analysis of the inhibition (not shown) indicates that H_2DIDS behaves as a non-competitive inhibitor with respect to ATP

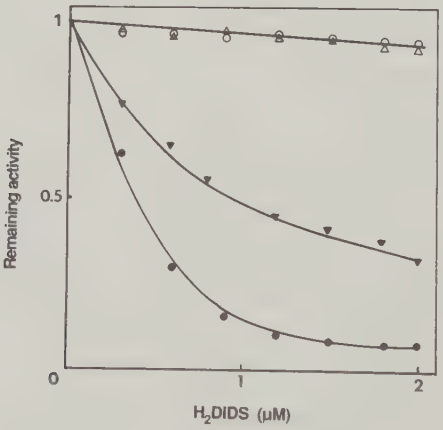

Figure 1: Reversible inhibition of Na,K-ATPase and pNPPase activities by H_2DIDS. Na,K-ATPase (▼) and pNPPase (●) activities were measured in the presence (○▲) and absence (●▲) of 0.3 mg/ml albumin.

and K^+. As far as we know, this is the first time an inhibitor is described that affects the pNPPase more than the Na,K-ATPase activity. The apparent inhibition constant (K_i) was 1 uM and 0.4 uM for the Na,K-ATPase and pNPPase activities, respectively. The presence of 50 mM 2-mercaptoethanol, which reacts with the isothiocyanate groups of H_2DIDS, did not appreciably affect the reversible inhibition. On the other hand, 0.3 mg/ml albumin in the assay medium totally protects the enzyme activity (Fig. 1). The presence of Na^+ or ATP in the assay medium of the pNPPase activity did not affect the magnitude of the inhibition of this activity by H_2DIDS (not shown).

The degree of reversible inhibition was dependent on the pH. Na,K-ATPase was less inhibited at higher pH; the residual activity was 25%, 40%, 64%, and 75% at pH 6.5, 7.2, 7.9 and 8.4, respectively, in the presence of 2 uM H_2DIDS.

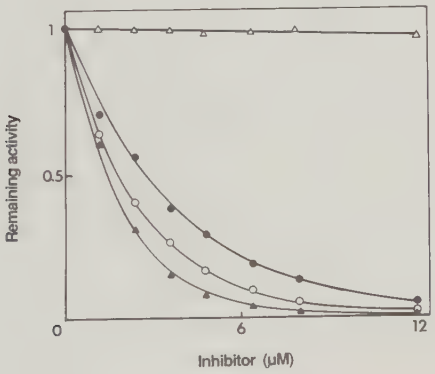

Figure 2: Irreversible inhibition of Na,K-ATPase by SITS, BIDS and H_2DIDS. The enzyme (0.033 mg/ml) was incubated at 37°C in the presence of 50 mM Tris HCl, 1 mM Tris-EDTA, pH (37°) 9 and different concentrations of SITS (●), BIDS (○) and H_2DIDS (▲) for one hour. The reaction was halted by addition of 50 mM 2-mercaptoethanol and 0.3 mg/ml albumin. Na,K-ATPase was then assayed. Also shown (△) is the effect of 50 mM 2-mercaptoethanol added to the reaction medium before H_2DIDS.

The inhibition of the pNPPase activity was less affected by the pH than the Na,K-ATPase activity (not shown).

Fig. 2 shows the irreversible inhibition of Na,K-ATPase activity by H_2DIDS, BIDS and SITS, at pH 9. This kind of inhibition was observed when the enzyme was incubated with the inhibitors in the absence of the enzyme ligands. pNPPase activity was inhibited to the same extent as the Na,K-ATPase activity (not shown). The presence of albumin protected the enzyme activity (Fig. 2).

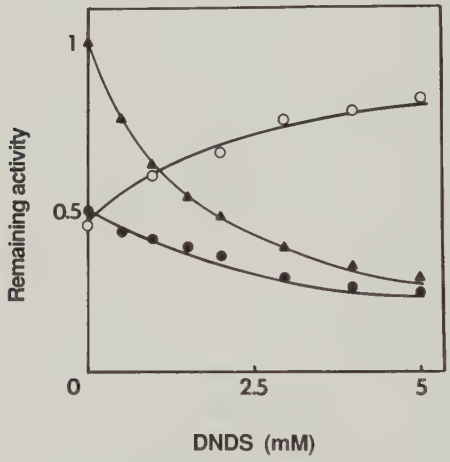

Figure 3: Effect of DNDS on the inhibition of Na,K-ATPase by H_2DIDS. The enzyme activity was assayed at different DNDS concentrations in the absence (▲) and presence (●) of 1 uM H_2DIDS. The protective effect of DNDS on the irreversible inhibition of the enzyme by H_2DIDS (O) was measured in 30 ul of a medium containing 50 mM Tris-HCl, 1 mM Tris-EDTA, pH (37°C) 7.2; 1 ug enzyme-protein, DNDS as indicated and 5 uM H_2DIDS and it was incubated at 37°C for 30 min. The reaction was stopped by addition of 20 ul of a solution containing 125 mM 2-mercaptoethanol and 0.75 mg/ml albumin. The enzyme activity was then assayed.

4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS), structurally related to H_2DIDS but lacking the isothiocyanate groups, produces only a reversible inhibition but protects the enzyme against the irreversible inhibition produced by H_2DIDS (Fig. 3). The data of Fig. 3 gave a very good fit to equations which describe competition between H_2DIDS and DNDS for the reversible interaction with the enzyme. Thus, DNDS protected the enzyme against H_2DIDS inactivation by blocking the reversible interaction of H_2DIDS with some domain in the protein necessary for the subsequent covalent attachment that resulted in irreversible inhibition. This suggests that the covalent reaction of H_2DIDS with the enzyme is modulated by a preceding reversible (electrostatic) interaction, at which stage the protection by DNDS is produced.

ATP (Kap.=90 uM) and K^+ (Kap.=80 uM) protect the Na,K-ATPase activity against the irreversible inhibition by H_2DIDS (data not shown). The binding of ADP (as an ATP analog) was reduced from 2.34±0.05 umol/mg in normal enzyme to 0.12±0.04 umol/mg in an enzyme inhibited 80% by H_2DIDS. However, the level of phosphorylation produced by inorganic phosphate (P_i) was not affected at all by the H_2DIDS treatment; 2.47±0.14 umol/mg and 2.67±0.12 umol/mg of P_i was bound to H_2DIDS-enzyme and control enzyme, respectively. The irreversible inhibition of the Na,K-ATPase by H_2DIDS followed first order kinetics with respect to time and inhibitor concentration. The level of H_2DIDS bound was 2.87±0.14 umol/mg when the enzyme activity was 23% of the control. These observations indicate that the inhibition is produced by the binding of about one H_2DIDS molecule per phosphorylation site.

Even though ATP or K^+ protect the enzyme against H_2DIDS inactivation, they do not prevent the H_2DIDS binding. Thus, in the presence of ATP or K^+, at concentrations which maximally protected the activity, H_2DIDS bound to the enzyme at a slightly higher level than that observed in the absence of these effectors when the activity is inhibited. However, it is clear that the binding site for H_2DIDS in the presence of K^+ or ATP is different from that observed in the absence of these ligands. Enzyme that was treated with H_2DIDS in the presence of ATP and conserved full activity could be inhibited by additional treatment with H_2DIDS in the absence

of the nucleotide. Thus, when the second treatment was made using [^{3}H]-H_2DIDS, 75% inhibition of activity was achieved with approximately 3 nmol/mg [^{3}H]-H_2DIDS bound. The protein electrophoretic pattern for enzyme that had been treated with H_2DIDS in the presence of either K^+ or ATP and main-

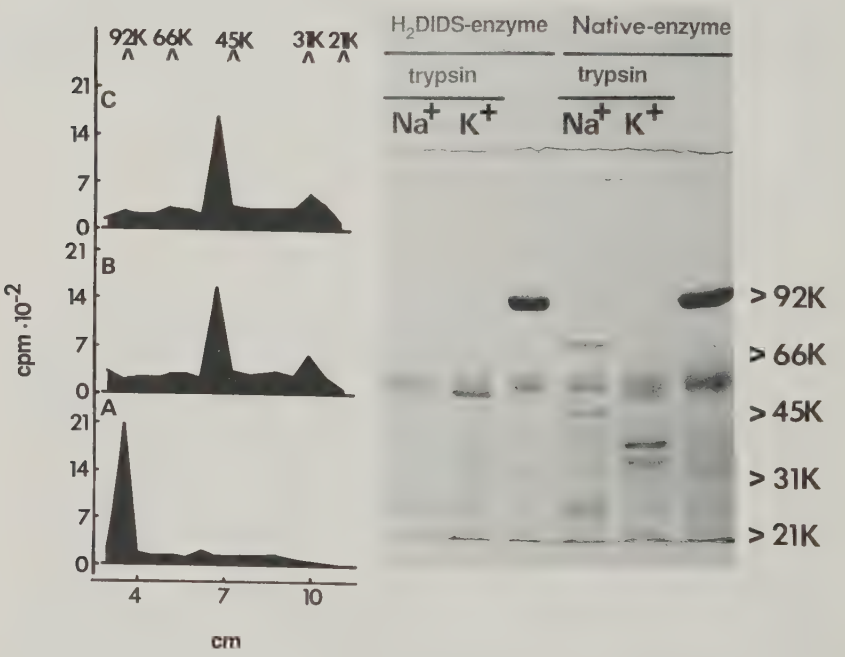

Figure 4: Left: ^{3}H-H_2DIDS labeling of the Na,K-ATPase. The gel whose picture is shown in the right was sliced, dissolved in H_2O_2 and the radioactivity counted. The picture shows the radioactivity profile of enzyme before (A) and after treatment with trypsin in the presence of either Na^+ plus ADP (B) or K^+ (C). Right: Polyacrylamide gel electrophoresis of trypsin treated H_2DIDS-enzyme. H_2DIDS-enzyme was treated with trypsin in the presence of either Na^+ plus ADP (lane Na^+) or K^+ (lane K^+) and subjected to SDS-PAGE on a 8-14% gradient gel. Na,K-ATPase not treated with H_2DIDS (native-enzyme) and H_2DIDS treated (H_2DIDS-enzyme) are shown. The third lanes of each are enzyme not treated with trypsin.

tained full activity was exactly the same as that of control enzyme, before and after trypsin treatment (not shown). More significantly, the degree of inhibition produced by the treatment with trypsin was the same in ATP- or K^+-protected enzymes as in native enzyme (data not shown).

SDS-PAGE of H_2DIDS irreversible inhibited enzyme indicated that the inhibitor covalently binds only to the α -subunit of the Na,K-ATPase (Fig. 4). Trypsin treatment of the H_2DIDS-labeled enzyme and subsequent analysis of the fragments by SDS-PAGE shows that the enzyme is kept in an E_2 conformation; thus the fragments produced by trypsin treatment in the presence of ADP plus Na^+ looked like they were produced in the presence of K^+ (Fig. 4). Even though some minor differences persisted like that of a small fragment of about 26K. The modified lysine residue is contained in the same 58KD peptide as the FITC-reactive lysine.

Comparing the fragmentation produced in the presence of K^+, two sharp bands of 38KD and 35KD in control enzyme are reduced in H_2DIDS-enzyme, while the major fragment of 52KD looks more intense. This might suggest that the 38KD and 35KD fragments are degradation product of the 52KD fragment and the presence of H_2DIDS on the protein reduces this proteolysis. Two very weak fragments of 45KD and 40KD are observed in H_2DIDS-enzyme and not in control enzyme. With respect to the trypsin degradation produced in the presence of Na^+ plus ADP, three major degradation fragments of 72KD, 47KD and 40KD are greatly reduced in H_2DIDS-enzyme. Other weak fragments as that of 45KD and 29KD are also reduced. A new fragment of 52KD, not observed in control enzyme, is present in H_2DIDS-enzyme.

H_2DIDS has two -NCS groups which could potentially react with different side chain $-NH_2$ residues. Covalent binding of both -NCS groups would result in the establishment of a cross-link. The possibility exists that it is not the reaction of the first -NCS group which produces the enzyme inactivation but the reaction of the second one. The observed inhibition might be due to either the establishment of the cross-link itself or the reaction of the second -NCS with an essential $-NH_2$ group. This mechanism appears not to apply to inactivation of the Na,K-ATPase by H_2DIDS because SITS and BIDS which have only one isothiocyano group

produced an irreversible inhibition of the Na,K-ATPase with kinetics which were very similar to that observed with H_2DIDS (Data not shown). No cross-linking of protein fragments in polyacrylamide gel electrophoresis (SDS-PAGE) of H_2DIDS-enzyme proteolyzed by trypsin was observed (see Fig. 4). However the possible formation of such cross-links, though unrelated to inhibition, might be revealed by more extensive proteolysis.

ACKNOWLEDGEMENT

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EXCHANGE AND HYDROLYSIS KINETICS OF Na,K-ATPase

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INTRODUCTION

Parallel measurements in steady state of the ATP hydrolysis rate (V_{hydr}) and the simultaneous reverse reaction, i.e. the ADP/ATP-exchange (V_{exch}), allowed the determination of a kinetic parameter, K_E, which can be shown to consist of only the four rate constants needed to characterize the enzyme intermediates involved in the sequence

$$\overset{-1}{\longleftarrow} EATP \underset{-2}{\overset{2}{\rightleftharpoons}} ADP + EP \overset{3}{\longrightarrow} \qquad K_E = \frac{v_{exch}}{v_{hydr}} \cdot \frac{1}{(ADP)} = \frac{k_{-2}}{k_3(1+k_2/k_{-1})} \cdot \frac{1}{(ADP)}$$

In order to compare the properties of these intermediates under different sets of conditions, K_E was measured at varying K^+-concentrations using enzyme partially purified from bovine brain.

EXPERIMENTAL PROCEDURES

Na,K-ATPase was prepared from bovine brain. The undiluted preparation contained 3.2 mg protein per ml. The activity was 4.4 μmol ATP hydrolysed per mg protein per min at pH 7.4 (37°C).

ATP hydrolysis rates were measured according to

Lindberg and Ernster (1956). ADP/ATP exchange was measured according to Randerath & Randerath (1967) as modified by Cashel et al. (1969). It was at all times ensured that the fastest of the two opposing reactions changed by less than 10% the concentration of the species MgATP or ADP that was present in the lowest concentration. All assays were performed at pH 7.4 and at 37°C. (Mg_{free}) was 100 µM in the reported experiments.

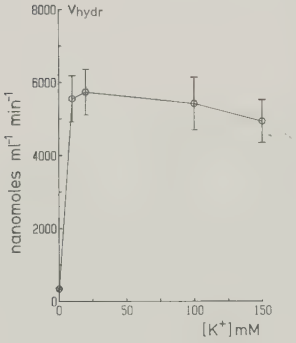


Fig. 1. ATP-hydrolysis rate vs. K^+-concentration. Na^+ 150 mM, Mg free 0.1 mM, MgATP 1.18 mM and ATP_{free} = ADP_{free} = 1 mM in a histidine buffer (30 mM) pH 7.4 at 37°C. The values are means of 3 experiments, and the length of the vertical bars are twice the standard deviation.

RESULTS AND DISCUSSION

In Fig. 1 is shown the well known activation by K^+ of Na,K-ATPase at high (1.18 mM) MgATP and 150 mM Na^+. The hydrolysis rate is maximal at about 25 mM K^+ with a small decrease at higher K^+ concentrations.

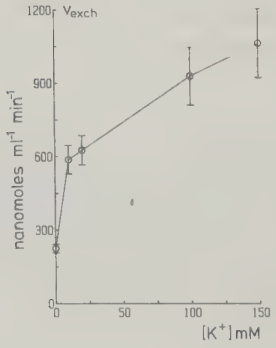
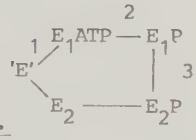


Fig. 2. ADP/ATP-exchange rate vs. K^+- concentration. The assay conditions were the same as in fig. 1. The values are means of 3 experiments and the length of the vertical bars are twice the standard deviations.

In Fig. 2 is shown the ADP/ATP exchange rate measured under the same conditions as those of Fig. 1. It is seen that the exchange rate continues to increase with K^+ concentrations up to 150 mM, showing no sign of decrease or levelling off in this range.

At high (millimolar) substrate concentrations the basic features of the Albers-Post model may be represented as shown in scheme 1, where the states E_1P and E_2P represent, respectively, the ADP sensitive and the K^+-sensitive phosphoenzymes that have to be traversed whether or not K^+ is present in the reaction mixture. 'E' designates the conformation E_1 in the absence of K^+ and the conformation $E_2(K)$ in its presence. In the latter case the transition 'E' -> E_1ATP then involves i) binding of ATP, ii) conformational change of E_2 to E_1, and iii) release of occluded K^+.



Scheme 1.

It may be shown, using conventional procedures for derivation of isotope exchange rates that the ADP/ATP exchange rate for scheme 1) is

$$v_{exch} = \frac{k_{-2}}{1+k_2/k_{-1}} \cdot (ADP)\cdot(E_1P) \qquad (1)$$

while the hydrolysis rate is given by

$$v_{hydr} = k_3\cdot(E_1P) - k_{-3}\cdot(E_2P) \qquad (2)$$

The individual rate constants in Eqs. (1-2) may depend on the concentrations of ligands interacting with the kinetic intermediates. Thus since K^+ binds to EATP in scheme 1 prior to the release of ATP from the enzyme in the backwards reaction, k_2 will decrease (to zero) and k_{-1} will increase (to a finite limit) as (K^+) increases. Since k_{-2} is not affected (K^+ does not bind to E_1P according to the Albers-Post scheme) the factor multiplying the concentration terms in Eq. (1) increases (to k_{-2}) as (K^+) is increased.

One feature in the Albers Post scheme that should be emphasized is that E_1P is the only intermediate with which K^+ does not directly interact. As the concentration of K^+ is increased we should therefore expect that the steady state concentration of E_1P decreases, possibly after a transient increase due to the expected decrease in the steady-state concentration of E_2P, at least if the substrate concentration is sufficiently high to prevent a piling up of $E_2(K)$. The expected decrease in (E_1P) at high (K^+) is reflected in Fig. 1 for $(K^+) > 25$ mM. At this K^+ concentration (E_2P) is expected to be virtually zero, and from Eq. (2) we then have $v_{hydr} = k_3(E_1P)$, from which it follows, with the data in Fig. 1, that (E_1P) decreases as (K^+) is increased. However, as seen in Fig. 2 the exchange rate continues to increase in this range. It follows that the exchange rate per se is not a good measure of the E_1P concentration. The observed increase in v_{exch} can only be explained in terms of the Albers Post scheme by the influence of K^+ on the rate constants, k_2 and k_{-1}, yielding an increase in the rate constant factor in Eq. (1) which more than compensates for the decrease in (E_1P).

If the back reaction in Eq. (2) can be ignored, we can eliminate (E_1P) between Eqs. (1) and (2) to obtain

$$\frac{v_{exch}}{v_{hydr}} = \frac{k_{-2}}{k_3(1+k_2/k_{-1})} \cdot (ADP) = K_E \cdot (ADP) \qquad (3)$$

which defines the exchange constant K_E. From the arguments above we would expect K_E, containing only rate constants emanating from the kinetic states EATP and E_1P, to be a monotonically increasing function of (K^+), and hence the value of K_E at $(K^+) = 0$ should be lower than any value obtained at finite (K^+). Values of K_E obtained from the experiments in Figs. (1) and (2) are shown as a function of (K^+) in Fig. 3.

Fig. 3. K_E vs. K^+-concentration. K_E is calculated on the basis of the data in Figs. 1 and 2.

The sharp increase observed as (K^+) approaches zero is not consistent with the above prediction, which in turn is based on the Albers-Post model. The behavior in Fig. 3 can be explained, however, if the set of rate constants making up K_E (Eq.(3)) in the presence of Na^+ alone is different from that when also K^+ is present, i.e. if the species EATP and E_1P are different in these two conditions.

It might be argued that in fact the back reaction at small or zero K^+ concentration cannot be ignored, and therefore the expression for K_E, Eq. (3), is not valid under these conditions. It appears from other work (Fortes et al. this volume) that at 37°C, the steady state concentration of E_1P in the absence of K^+ and at saturating ATP is 13-25% of the total enzyme concentration, the remaining part being mainly E_2P. It follows that k_3 and k_{-3} must be of the same order of magnitude (with $k_3 > k_{-3}$) for the back reaction to make a significant contribution. Both must then be of the order of the turnover for the Na,K-ATPase in the presence of K^+ in order to account for the high activity observed under these conditions. If so, E_1P and E_2P must be in rapid equilibrium at steady state in the absence of K^+. In that case, addition of K^+ or ADP to enzyme phosphorylated in the presence of Na^+ alone should rapidly remove all phosphoenzyme, contrary to the well known biphasic behavior observed in both cases. We therefore consider it most likely that the intermediates EATP and E_1P and their associated rate constants are different depending on whether or not K^+, in addition to Na^+, is present during the steady state turnover, and that a complete model for Na,K-ATPase must contain two distinct hydrolysis cycles, (Plesner and Plesner, 1987 a,b.)

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DEPHOSPHORYLATION SCHEMES FOR THE Na,K-ATPASE RECONSIDERED IN LIGHT OF ALLOSTERIC SITES FOR Na

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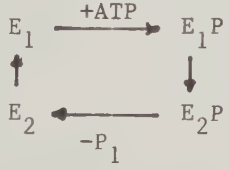
Central to various Albers-Post schemes for the Na,K-ATPase reaction sequence is the cycle



with reversible transphosphorylation and dephosphorylation in the horizontal steps and transport in the vertical steps. This scheme specifies that E_1P is subject to transphosphorylation (to re-form ATP) but not hydrolysis (to release P_i), whereas E_2P is subject to hydrolysis following the transport of Na; as Jencks (1983) and Tanford (1983) have emphasized, such a sequence avoids dissipation of ATP group transfer free energy in the absence of transport, thereby assuring energy coupling.

Recent criticisms of the Albers-Post schemes include: (a) demonstrations that the sum of ADP-sensitive phosphoenzyme ("E_1P") plus K-sensitive phosphoenzyme ("E_2P") increases well past 100% of total phosphoenzyme as the medium Na-concentration is raised (e.g., Norby et al., 1983; Yoda and Yoda, 1986); and (b) arguments from the slope of the slow phase of ADP-dependent dephosphorylation and from the relative rates of Na- and of K-activated dephosphorylations that E_1P is not an intermediate in the Na,K-ATPase reaction (Klodos et al., 1981; Plesner et al., 1981).

Instead, Norby et al. (1983) proposed a minimal model with three sequential phosphoenzymes, $E_1 \sim P$, E_1-P, and E_2-P, each of which is subject to hydrolytic dephosphorylation. The model fits the ADP- and K-sensitive dephosphorylation data admirably. However, it leads to two disquieting conclusions: (a) the energy-conserving feature of the Albers-Post scheme is lost, and (b) E_1-P is no longer an intermediate in the Na,K-ATPase reaction.

Hypotheses are inevitably underdetermined by the evidence and apparent anomalies can often be resolved by modest reformulations. To rescue the Albers-Post scheme in plausible form requires considering how the higher Na concentrations (responsible for "E_1P" plus "E_2P" totalling more than 100% of EP) affect the enzyme. Early experiments (Robinson, 1967) suggested heterotropic allosteric effects of Na, and numerous studies have shown specific effects of higher concentrations of extracellular Na on enzyme properties. Particularly pertinent are demonstrations of allosteric effects of extracellular Na

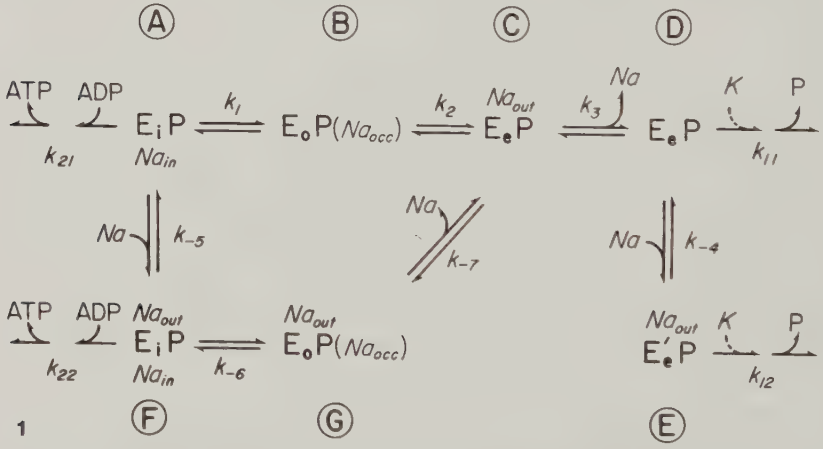


Fig. 1. Proposed scheme with Na binding to extracellular sites. "E_iP" refers to phosphoenzyme with acceptance sites for Na accessible in situ from the intracellular medium; "E_oP" to phosphoenzyme with Na at occluded sites; "E_eP" to phosphoenzyme with discharge sites for Na accessible in situ to the extracellular medium; and "E'_eP" to phosphoenzyme with acceptance sites accessible in situ to the extracellular medium at which K (or Na) can activate dephosphorylation (thus Na at E_eP drives the reaction to the left, Na at E'_eP drives it to the right).

on K-influx (Sachs, 1977) and on Na-extrusion (Karlish and Stein, 1985), as well as of high Na concentrations both driving the reaction back toward E_1P and forward toward dephosphorylation of E_2P (e.g., Hara and Nakao, 1981).

A plausible alternative to the Norby model thus would include extracellular sites (perhaps aspects of the transport channels) at which Na can affect both "E_1P" and "E_2P". To evaluate quantitatively a minimal model with such sites (Fig. 1), values for the parameters were selected empirically. First, the steady state

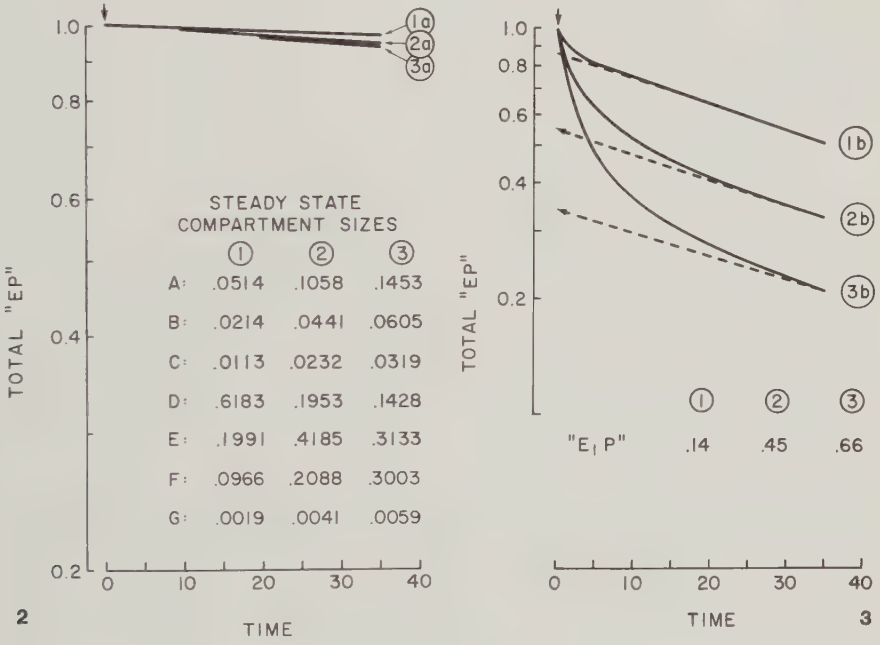


Fig. 2 (left). Dephosphorylation in absence of ADP and of K: with the parameters listed in Table 1 for cases 1a, 2a, and 3a, the total EP then decays as shown when phosphorylation is halted (arrow).

Fig. 3 (right). Dephosphorylation in presence of ADP: with the parameters listed for cases 1b, 2b, and 3b, total EP decays as shown. The tangents extended to the ordinate indicate ADP-sensitive phosphoenzyme, "E_1P".

concentration of each phosphoenzyme form for cases 1, 2, and 3 was calculated, using parameters 1a, 2a, and 3a (Table 1) and the steady state equations. Next, the decay of each of these forms was calculated: either from the cessation of further phosphorylation (Fig. 2) representing Na-ATPase activity, or from the cessation of further phosphorylation plus the addition of ADP (Fig. 3) or plus the addition of KCl (Fig. 4). The solutions to the differential equations were approximated by iterative evaluations in steps of Δt.

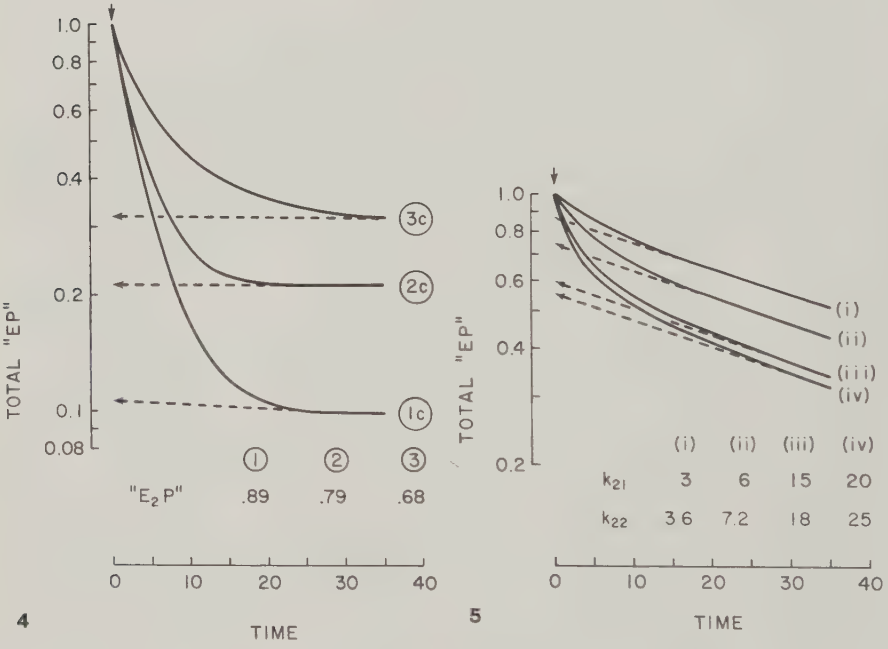


Fig. 4 (left). Dephosphorylation in presence of K: with the parameters listed for cases 1c, 2c, and 3c, total EP decays as shown. The tangents extended to the ordinate indicate K-sensitive phosphoenzyme, "E_2P".

Fig. 5 (right). Dephosphorylation in presence of increasing concentrations of ADP: with the parameters of case 2b, but with k_{21} and k_{22} varied as listed to represent increasing concentrations of ADP, total EP decays as shown, with parallel tangents to the slow phase.

TABLE 1. Parameters for Reaction Scheme in Figs. 2-4.

Parameter	Case								
	1a	b	c	2a	b	c	3a	b	c
k_1	100			100			100		
k_{-1}	240			240			240		
k_2	100			100			100		
k_{-2}	190			190			190		
k_3	145			145			145		
k_{-3}	2.6			17			32		
k_4	0.19			1.35			1.47		
k_{-4}	0.55			0.55			0.55		
k_6	0.09			0.09			0.09		
k_{-6}	4.55			4.55			4.55		
k_7	0.59			0.59			0.59		
k_{-7}	0.10			0.105			0.11		
k_{11}	0.03	0.03	10	0.06	0.06	10	0.09	0.09	10
k_{12}	0.04	0.04	12	0.08	0.08	12	0.12	0.12	12
k_{21}	0	20	0	0	20	0	0	20	0
k_{22}	0	25	0	0	25	0	0	25	0

TABLE 2. Comparison of Proposed Scheme with Data.

Form	Reaction Scheme			Data (Norby et al.(1983)) with [NaCl] of:		
	Case 1	Case 2	Case 3	20 mM	150 mM	300 mM
"E_1P"	.14	.45	.66	.15	.45	.67
"E_2P"	.89	.79	.68	.88	.81	.68
Sum	1.03	1.24	1.34	1.03	1.26	1.35

This scheme thus functions for both Na- and Na,K-ATPase reactions and replicates the values of Norby et al. on "E_1P" plus "E_2P" increasing well beyond 100% (Table 2). Moreover, the slow phases of ADP-dependent dephosphorylation, as a function of [ADP], are parallel (Fig. 5), as observed by Klodos et al. (1981). This scheme requires no novel reaction intermediates or conformations. It is, however, an oversimplification, including neither multiple sites for Na and for K nor the liklihood that K could occupy extracellular sites on E_iP

and $E_o P$. The ratio of hydrolysis in the steady-state with Na plus K to that with Na alone has not been examined, although attention to the relative magnitudes of k_{11} and k_{12} vs. k_{-5} and k_7 should satisfy that requirement. The potential for high Na concentrations inhibiting the slow phase of dephosphorylation (particularly that in the presence of K) has also not been evaluated, but would seem to follow from effects through k_5 and k_{-7}.

CONCLUSIONS

1. The proposed reaction scheme depicts plausible roles for Na at higher concentrations, imposed upon a standard Albers-Post reaction sequence (Fig. 1).

2. Such a scheme can permit the sum of ADP-sensitive phosphoenzyme ("E_1P") plus K-sensitive phosphoenzyme ("E_2P") to exceed 100% of total EP, and quantitative evaluation (Table 1) of the parameters in Fig. 1 reproduce the data of Norby et al. (1983) on "E_1P" and "E_2P" contents with 20, 150, and 300 mM NaCl (Figs. 2-4; Table 2).

3. Moreover, the proposed scheme also reproduces (Fig. 5) the data showing parallel slow phases of dephosphorylation as ADP is increased, in contrast to standard Albers-Post schemes (Klodos et al., 1981).

4. Thus an Albers-Post scheme modified to include plausible interactions with extracellular Na retains salient features of the original:
    a) The phosphoenzymes are intermediates of both Na- and Na,K-ATPase pathways; and
    b) Energy coupling is assured by interposing the transport step between hydrolysis-resistant $E_i P$ and hydrolysis-sensitive $E'_e P$.

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A COMPARISON BETWEEN THE EMPIRICAL BEHAVIOUR OF THE Na,K-ATPase AND THE PREDICTIONS OF THE ALBERS-POST MODEL

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INTRODUCTION

In previous communications we have reported that, when the high-affinity component of the substrate curve of the Na,K-ATPase is fully saturated, changes in the concentration of K or of ATP alter in the same proportion the apparent affinities of the Na,K-ATPase for these ligands and their maximum effects (Garrahan, et al., 1982, see also Eisner and Richards, 1981). In constrast with this, when the concentrations of either ATP and Na or of ATP and Mg are modified, maximum effects are altered but apparent affinities remain constant (Garrahan et al., 1982).

Several kinetic equations account equally well for this behaviour. To discriminate among them it is necessary to study the interactions between Mg, K and Na during Na,K-ATPase activity. In this communication we report results of such a study and we show that when these results are taken together with those mentioned above it is possible to derive an equation that adequately describes the activation by cations of the Na,K-ATPase. This equation allowed us to compare the behaviour of the enzyme with the predictions of the Albers-Post scheme and to show that some of the predictions of this scheme are difficult to reconcile with the observed behaviour of the Na,K-ATPase.

Partially purified Na,K-ATPase (Joergensen, 1974), from dog kidney was used. The experimental conditions and the procedure to fit non-linear equations to the data have been described elsewhere (Rossi and Garrahan, 1985).

RESULTS

Interactions between Mg and Na.

Na,K-ATPase activity was measured as a function of the concentration of free Mg ions in media with 3 mM total ATP, 10 mM K and different concentrations of Na. The equation that gave best fit to the data was:

$$v = Vmax / ((1 + f(Na)) (1 + KO.5/[Mg\ free])) \quad (1)$$

Where f(Na) is a function of the concentration of Na whose value varies from infinity to zero as the concentration of Na goes from zero to infinity (see below).

Interactions between Mg and K.

Na,K-ATPase activity was measured as a function of the concentration of free Mg ions in media with 3 mM total ATP, 120 mM Na and different concentrations of K. The equation that gave best fit to the data was:

$$v = Vmax / (1 + f(K) + KO.5/[Mg\ free]) \quad (2)$$

Where f(K) has the same meaning as f(Na) in eqn. (1) (see below).

Interactions between Na and K.

This study proved to be difficult because of the sigmoid shape of the functions that relate activation to the concentrations of Na or of K and of the competitive interactions between these cations. Non-linear regression showed that the best-fitting equation for activity vs. Na concentration at a given concentration of K and at non-limiting concentrations of the rest of the ligands was:

$$v = Vmax / (1 + KNa/[Na]^2) \quad (3)$$

Eqn. (4) gave a better description of the kinetics of activation than the ratio between Vm and a polynomial of third degree in Na concentration or than the commonly used expression (Garay and Garrahan, 1973):

$$v = Vmax / (1 + KNa/[Na])^3 \quad (4)$$

Multiple regression analysis of ATPase activity as a function of the concentrations of both Na (in a 0 to 5 mM concentration range) and K (in a 0 to 1 mM concentration range) at non-limiting concentrations of the other ligands, yielded the following expression as the best-fitting equation:

$$v = \frac{Vmax}{1 + \frac{KK1}{[K]}\left(1 + \frac{[Na]}{KiNa}\right) + \frac{KK2}{[K]^2}\left(1 + \frac{[Na]}{KiNa}\right) + \frac{KNa}{[Na]^2}} \quad (5)$$

No significant improvement in the goodness of fit was obtained by including squared inhibitory terms and/or competition by K of the activation by Na.

DISCUSSION

When our previous results and those shown here are taken together, the following equation seems to give an adequate description of the interactions between the Na,K-ATPase and a pair of activating ligands at non-limiting concentrations of the rest and when the high-affinity component of the substrate curve of the enzyme is saturated:

$$v = Vmax / ((1 + f(ATP))\ (1 + f(Na))\ (1 + f(Mg)) + f(K)) \quad (6)$$

The functions of the cations are those in eqns. (1) to (5). The shape of f(ATP) has been discussed previously (Rossi and Garrahan, 1985).

To see if the behaviour described by eqn. (6) is the predicted by the Albers-Post model, we performed computer simulations of the effects of ligands on the steady-state behaviour of this model. To do this we solved the kinetic scheme in Fig. (1) for initial velocity in the absence of products. Cations were assumed to bind in rapid-equilibrium so that the values of the rate constants can be expressed as functions of the concentration of the cations. For the sake of simplicity we assumed that the functions were hyperbolic. This does not affect the generality of the treatment. On the basis of the usual tenets of the Albers-Post model the

following assignments were made for the effects of cations on the rate constants:

$$k2' = k2max[Na][Mg]/(KNaKMg + KNa[Mg] + KMg[Na] + [Na][Mg]) \quad (7)$$

$$k4' = k4max[K]/([K] + KK) \text{ and } k_3 = k_3max/([K] + KK) \quad (8)$$

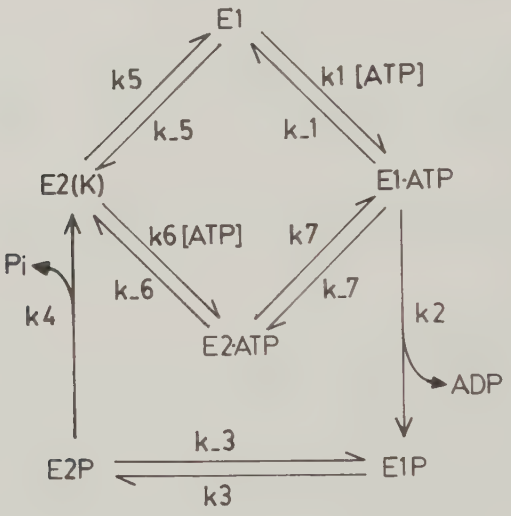

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Figure 1. Reaction scheme for the hydrolysis of ATP by the Na,K-ATPase. (based on Moczydlowski and Fortes, 1981)

The values of the rate constants were taken from the literature (Moczydlowski and Fortes, 1981 and Rossi and Garrahan, 1985). Dissociation constants for cations were arbitrarily set at 2 mM. The concentration of the cation under test was varied from zero to at least 20 mM, in order to attain at least 90% saturation.

The simulation adequately predicted the experimental behaviour for the interactions between K and any of the ligands tested. In all of these maximum effects and apparent-affinities are altered in the same proportion. This behaviour indicates the operation of either ping-pong or ordered sequential mechanisms with the binding of ligands separated by at least one irreversible step. The Albers-Post

scheme assumes ping-pong mechanisms between K and Na and between K and Mg and sequential mechanisms between K and ATP, the binding of ATP to E2 being separated from that of K by a step that is irreversible in the absence of Pi. This explains the agreement between the experimental and the simulated behaviour.

For the interactions that do not involve K, in all cases the experiments indicated that only maximum effects were altered. This contrasted with the simulation of the Albers-Post scheme which predicted proportional modifications of maximum effects and apparent affinities for the interactions between ATP and Na and between ATP and Mg, and large possitive effects on apparent affinities for the interactions between Na and Mg. For the interactions between Na and ATP and between Mg and ATP, the cause of the discrepancy is that the scheme implies ping-pong or sequential mechanisms respectively. In the case of the interactions between Mg and Na the discrepancy results from the fact that the scheme assumes random binding of these cations to E1ATP (Mardh and Post, 1977) which yields possitive interactions in apparent affinities due to the presence of steps that do not need the binding of Na or Mg.

To see if the Albers-Post scheme can be modified to account for the observed discrepancies, we performed simulations assuming that other constants apart from k2 were dependent on Na and Mg. Only k5 and k7 may be modified by these cations. If other steps are altered by Na and Mg the agreement between the experimental and the simulated behaviour in what concerns the interactions between K and the other ligands is lost. The simulations showed that if random rapid-equilibrium binding of Na and Mg were required for the E2 to E1 and the E2ATP to E1ATP transitions, the apparent affinity for any of the ligands involved would not vary by more than 30% as the concentration of one of the other two ligands were varied from zero to infinity. Hence under this hypothesis the simulated response approaches the experimentally observed behaviour. It must however be stressed that studies on partial reactions (see Glynn, 1985) seem to indicate that the E2 to E1 transitions do not require Na or Mg. It might be argued that (Na + Mg)-dependent steps in the E2 to E1 transitions are not detected when the transitions are measured as partial reactions under zero turnover conditions. Nevertheless until such hypothesis is proved, the possibility that there is an intrinsic flaw in

the Albers-Post scheme cannot be discarded.

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PHOSPHORYLATION OF (Na^++K^+)-ATPase. STIMULATION AND INHIBITION BY SUBSTITUTED AND UNSUBSTITUTED AMINES

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INTRODUCTION

In the view of a previously established stimulation of steady-state phosphorylation of (Na^++K^+)-ATPase by imidazole and its inhibition by tris-(hydroxymethyl)-aminomethane (Schuurmans Stekhoven et al., 1985) the effect of structure, chemical composition and charge of a number of primary, secondary and tertiary amines has been investigated.

EXPERIMENTAL

The enzyme has been isolated and purified from rabbit kidney outer medulla according to the procedure of Jørgensen (Jørgensen, 1974). Phosphorylation (3 s) in the control assay occurs in 50 mM imidazole-HCl, pH 7.0, at 23°C and the presence of 0.1 mg protein/ml, 0.1 mM $MgCl_2$ and 20 µM [γ-^{32}P]ATP (imidazole-salt). This yields an average level of 50% (range 40-60%) of the maximal Na^+-activated phosphorylation. The amino compounds (HCl-salts, pH 7.0) are assayed in the above medium in the 5-150 mM concentration range (the strongly inhibitory diamines in the 0.02-20 mM concentration range) and their phosphorylation expressed as ratio of the control.

RESULTS

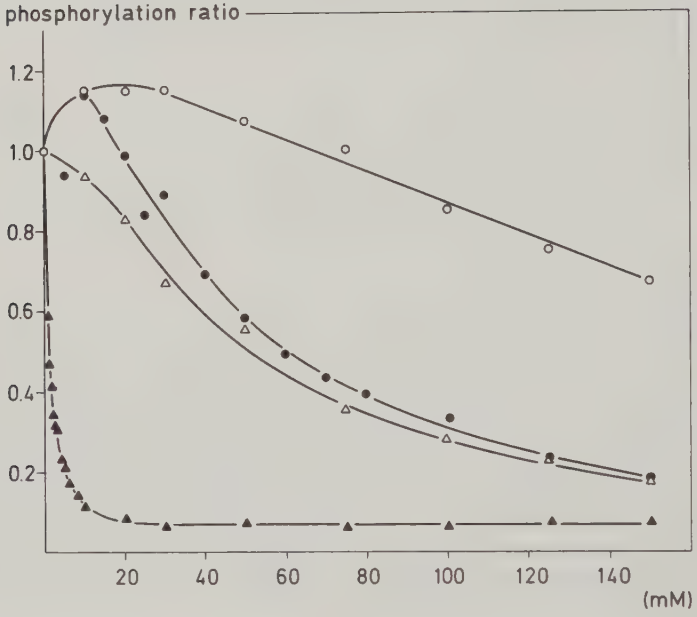


Fig. 1. Primary amines are predominantly inhibitory, following a transient stimulation at 10-30 mM as seen for 3-NH_2-pyridine (o) and n-propylamine (●). The $(CH_2OH)_3C$ substituted Tris (△) does not show any stimulation and the primary diamine 2-NH_2-ethyl-trimethyl ammoniumchloride (H_2N-$(CH_2)_2$-$N(CH_3)_3Cl$, ▲) shows only strong inhibition (I_{50} = 0.8 mM).

Secondary amines of comparable chemical nature display less inhibitory power, not necessarily a stronger stimulatory potency (Fig. 3).

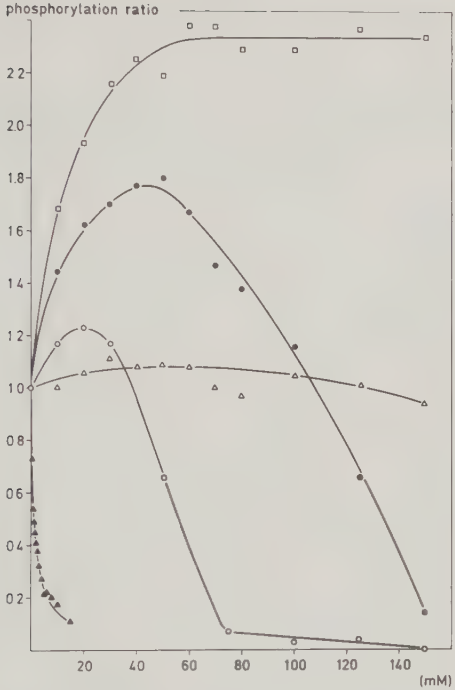
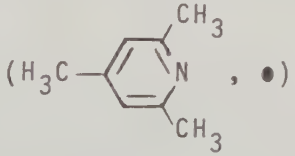
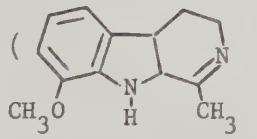


Fig. 2. Tertiary amines display a strong variability of effects, from strong stimulation by triallylamine ((CH_2=CH-CH_2)$_3$N, □), via intermediate stimulation by the shielded heterocyclic N-compound 2,4,6-trimethyl pyridine



and weak or no stimulation by the unshielded 4-ethylpyridine (○) and triethanolamine ((C_2H_5OH)$_3$N, Δ), to strong inhibition by the ethylene diamine derivative harmaline



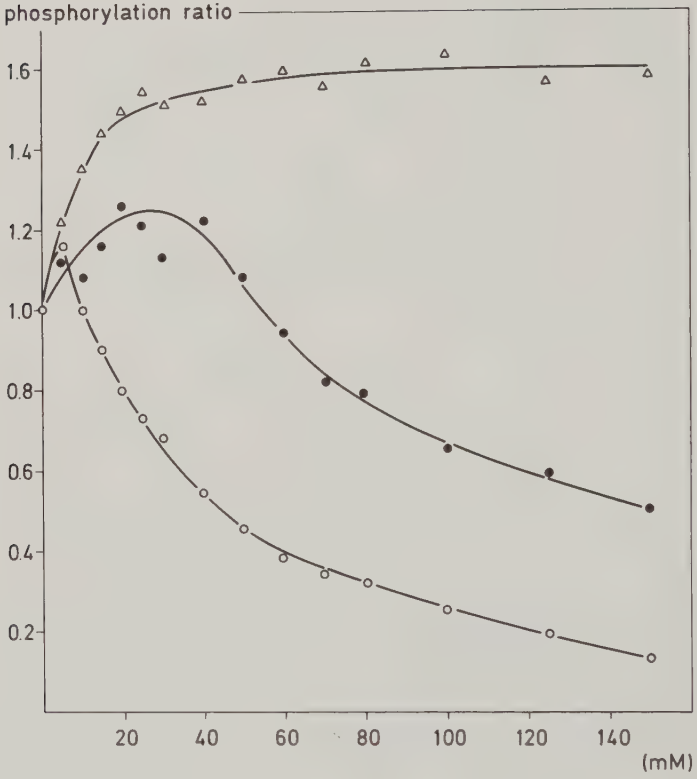


Fig. 3. The effect of increasing alkyl substitution on the phosphorylation level. Increasing the extent of substitution by allyl (CH_2 = CH-CH_2)-groups from mono-(o) via di-(•) to triallylamine (Δ) increases the phosphorylation ratio from 1.2 to 1.6 and abolishes inhibition. Substitution by propyl groups gives similar results. Tetrapropyl substitution (max. phosphorylation ratio 1.4) is inferior to tripropyl substitution (max. ratio 1.6).

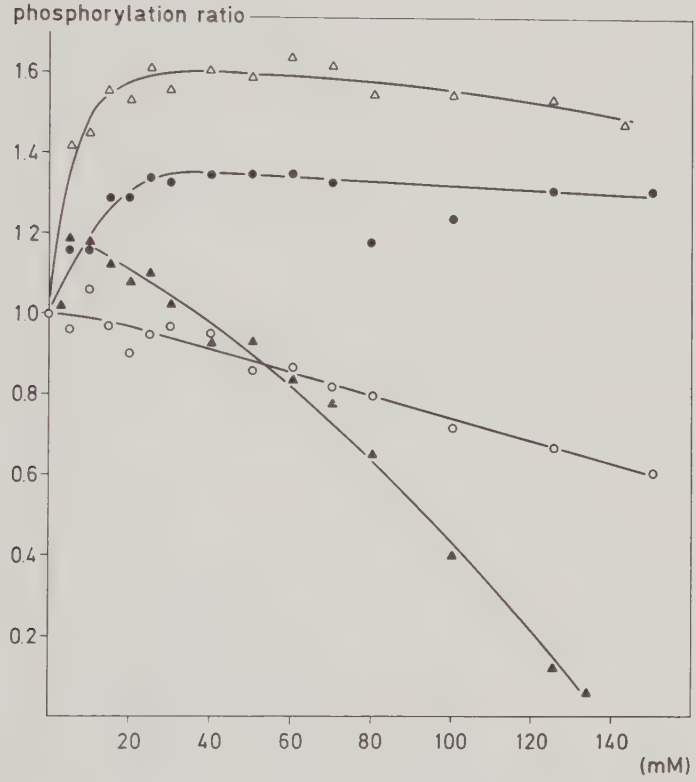


Fig. 4. The effect of the alkyl chain length in trialkyl substitution. Increasing the size of the substituents from CH_3 (o) via C_2H_5 (•) to C_3H_8 (Δ) increases the phosphorylation ratio to 1.6. A further increase to C_4H_{10} (▲) again decreases the ratio and leads to inhibition.

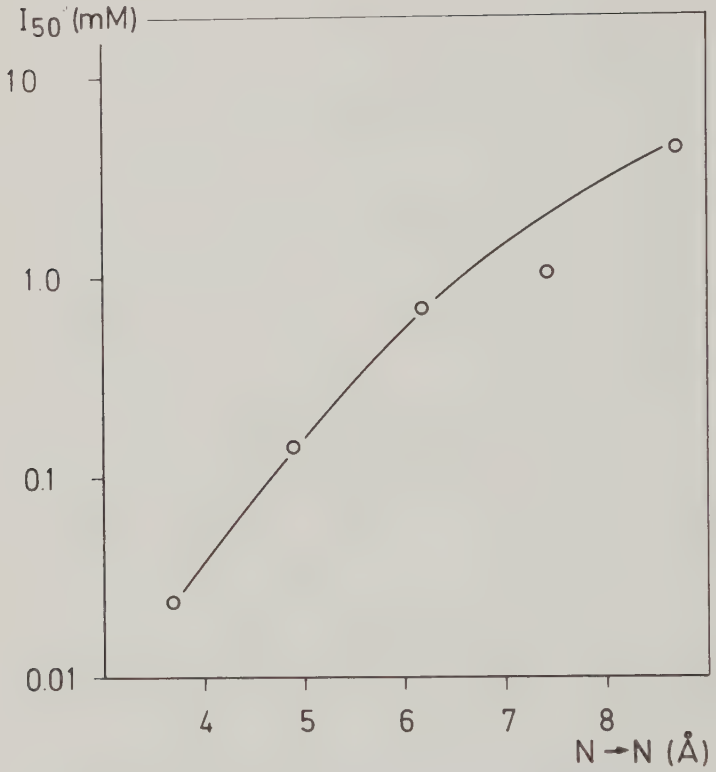


Fig. 5. Effect of increasing the N → N distance in alkyl-diamine ($H_2N-(CH_2)_n-NH_2$, n=2-6) on inhibition (I_{50}). Increasing the distance from 3.7 (ethylene diamine) to 8.7 Å (1,6-diaminohexane) increases the I_{50}-value 180-fold (0.025 to 4.5 mM).

The inhibition by ethylenediamine of Na^+-activated phosphorylation is competitive towards Na^+ but inhibition of triallylamine activation is mixed type (the half-maximally activating concentration of the buffer increases, while the maximal phosphorylation level decreases at increasing the inhibitor concentration, Table 1). Likewise, activation by Mg^{2+} and ATP in triallylamine buffer is inhibited mixed type.

TABLE 1. Inhibition of Na^+- or triallylamine-activated phosphorylation by ethylene diamine (EDA).

Na^+ (0-20 mM)-activated phosphorylation takes place in 50 mM triethanolamine-HCl at pH7.0, and triallylamine (0-150mM)-activated phosphorylation in triallylamine-HCl, pH7.0, as the only buffer component. Phosphorylation is to steady-state, taking 3-15s in the presence of Na^+ or triallylamine but absence of EDA and 1-2 min in the presence of triallylamine and EDA. The phosphorylation in triallylamine follows a 45 min period of preincubation of enzyme in this buffer medium and an additional 2 min after addition of EDA, yielding equilibrium enzyme-inhibitor complexation.

Na^+-activated phosphorylation				Triallylamine-activated phosphorylation			
EDA (mM)	$[Na^+]0.5$ (mM)	EPmax (nmol.mg^{-1} protein)	(%)	EDA (mM)	[TAA]0.5 (mM)	EPmax (nmol.mg^{-1} protein)	(%)
0	0.2	2.53	100	0	8	2.58	100
0.35	0.9	2.46	97	0.2	37	2.23	86
0.7	1.55	2.48	98	0.4	47	1.86	72
1.5	1.9	2.44	96	0.6	55	1.56	60
2.1	2.6	2.41	95				

Effect of EDA on Mg^{2+}-and ATP-activation

Phosphorylation takes place in 150 mM triallylamine-HCl, pH7.0, containing either 0.1 mg protein/ml, 0.2 mM EDTA, 0.5-100 µM uncomplexed Mg^{2+} and 20 µM [γ-^{32}P]ATP or 0.03 mg protein/ml, 0.1 mM $MgCl_2$ and 0.1-5 µM [γ-^{32}P]ATP

EDA (mM)	Km Mg^{2+} (µM)	EPmax (nmol.mg^{-1} protein)	(%)	EDA (mM)	KmATP (µM)	EPmax (nmol.mg^{-1} protein)	(%)
0	0.19	2.04	100	0	0.11	2.18	100
0.6	0.53	1.54	75	0.6	0.45	1.38	63

CONCLUSIONS

1. Increasing alkyl-substitution of the central N-atom converts inhibition to stimulation. Optimal stimulation occurs by tripropyl- or triallylamine. Presumably lipophylic Van der Waals interaction in the active centre decreases entry of water and hence phospho-enzyme hydrolysis.
2. Substitution by hydrophylic groups, like in tris-(hydroxymethyl)-aminomethane and triethanolamine, does not prevent water entry to the hydrolytic centre and abolishes stimulation.
3. Ethylene diamine derivatives (N→N distance 3-4 Å) invariably demonstrate a strong inhibitory potency. Increasing the distance increases the I_{50}-value. Presumably cation activation sites, 3-4 Å apart, are involved in the binding of ethylene diamine.
4. The competitive inhibition by ethylene diamine of Na^+ activation vs. the mixed type inhibition of amino compound activation is another indication of different mechanisms or sites of activation. The allosteric Na^+ activation by imidazole (Schuurmans Stekhoven et al., 1985) is a previous example, in addition to interaction between these sites. The mixed type inhibition of triallylamine-activated phosphorylation proves that the buffer-enzyme-inhibitor complex is still partially productive.

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Pb2+ ACTIVATED ATP-PHOSPHORYLATION AS A TOOL TO DETERMINE THE E1/E2 RATIO OF Na,K-ATPase.

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INTRODUCTION

Recently, we have reported that imidazole activates the steady-state ATP phosphorylation (Schuurmans Stekhoven et al., 1985) of Na,K-ATPase via a reduction of the K-sensitivity of the phosphointermediate (Schuurmans Stekhoven et al., 1986). As Pb2+ is also an activator of the steady-state phosphorylation (Siegel and Fogt, 1976), we have compared the effects of imidazole and Pb2+ on the phosphorylation and dephosphorylation of Na,K-ATPase (Swarts et al., 1987). The results indicate that Pb2+ activated ATP phosphorylation could be a tool to study the E1-E2 transition of Na,K-ATPase.

MATERIALS AND METHODS

Na,K-ATPase, purified from rabbit kidney outer medulla (Jørgensen, 1974), is phosphorylated in a medium containing 50 mM imidazole, 0-5 mM Mg2+, 0 or 100 mM Na^+, 0-1 mM Pb2+ and 5 µM [γ32P]ATP pH 7.0, at room temperature or at 0 C. After 3-60 s the reaction is stopped by acidification (Schuurmans Stekhoven et al., 1985) or used for dephoshorylation studies.

Dephosphorylation is performed by adding 0.5 mM ATP to the phosphorylation medium and it is terminated after 3 s incubation. The acid stable phosphointermediate is determined as decribed before (Schuurmans Stekhoven et al., 1986).

E-P generated in the absence of Pb2+ is called E-P.Mg and in its presence E-P.Pb.

RESULTS

Effect of Pb2+ on the steady-state phosphorylation.

In the absence of Na^+, Pb2+ increases (like imidazole) the steady-state ATP phosphorylation of Na,K-ATPase (Fig. 1A). High Pb2+ concentrations decrease the phosphorylation-level, and this inhibition of Pb2+ is counteracted by Mg2+ (Fig. 1B). Ki for Pb2+ = 1 µM and the Km for Mg2+ = 0.03 mM. In the presence of Na^+, where the phosphorylation level is maximal, also an inhibition by Pb2+ of the steady-state phosphorylation is observed. Again this effect is counteracted by Mg2+ (Fig. 1C).

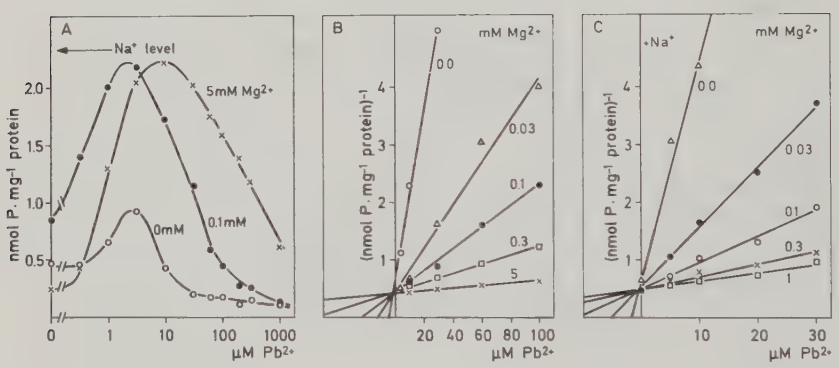

Figure 1A. The effect of Pb2+ and Mg2+ on the steady-state phosphorylation of Na,K-ATPase (0.1 mg/ml) in the presence of 50 mM imidazole and 5 µM [γ32P]ATP pH 7.0 at 20 C.
Figure 1B. Dixon plot of data from fig 1A.
Figure 1C. Dixon plot of the effect of Mg2+ on the inhibition of the steady-state phosphorylation by Pb2+ in the presence of 100 mM Na^+, 5 µM [γ32P]ATP, 50 mM imidazole, pH 7.0 and 0.1 mg/ml protein.

Effect of Pb2+ on the ATP phosphorylation rate constant.

For measuring the phosphorylation rate constant manually we lowered the temperature to 0 C, since at room temperature already a steady-state phosphorylation level is reached in 3 seconds. Fig. 2A and 2B show that upon addition of Pb2+ to the reaction medium the phosphorylation

rate constant increases from 0.08 per sec in the absence to 0.29 per sec in the presence of 3 µM Pb2+. The Km for this activating Pb2+ effect is 3 µM and is not affected by decreasing the Mg2+ concentration from 5 mM to 0.1 mM (Fig. 2C). This indicates that beside an inactivating site for Pb2+ (see previous section), also an activating site for Pb2+ is present on Na,K-ATPase which is not influenced by Mg2+.

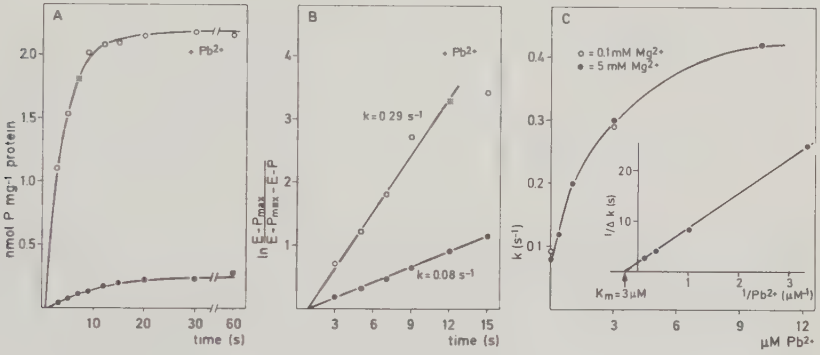

Figure 2. Effect of Pb2+ on the phosphorylation rate constant of Na,K-ATPase at 0 C, with 50 mM imidazole pH 7.0, 5 µM [ɣ32P]ATP, 5 mM Mg2+ (except open circles in part C), 0.1 mg/ml enzyme and the Pb2+ concentrations as indicated.

K-(in)sensitivity of the phospho-enzyme.

After phosphorylation of Na,K-ATPase by ATP in the absence of Na^+ and Pb2+ the phosphointermediate (E-P.Mg) is very sensitive to K^+ (Km = 4 µM in the presence of 50 mM imidazole, see also Schuurmans Stekhoven et al., 1986). Phosphorylation in the presence of 1 µM Pb2+ yields an intermediate (E-P.Pb) which is completely insensitive to K^+ (Fig. 3A).

Increasing the Mg2+ concentration during phosphorylation in the presence of Pb2+ does not change the K^+ insensitivity of the formed phosphointermediate (Fig. 3B).

Increasing the Na^+ concentration during phosphorylation in the presence of Pb2+ however, changes the K^+ sensitivity of the phosphoenzyme indicating that an E-P.Mg phosphointermediate is formed again (Fig. 3C).

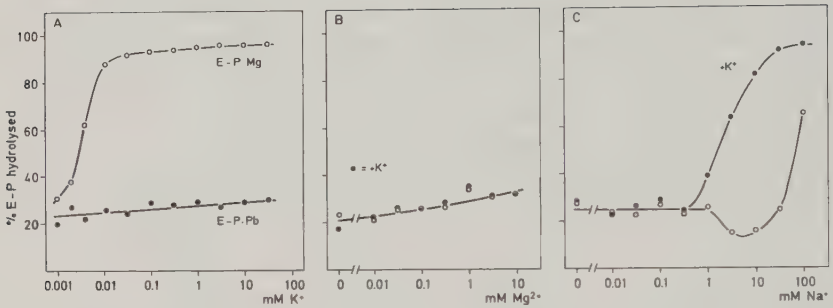

Figure 3A. Effect of K^+ on the stability of the phosphoenzyme generated with Mg2+ (E-P.Mg) or Pb2+ (E-P.Pb). After phosphorylation for 10 s at 20 C in a medium containing 5 µM [γ32P]ATP, 0.1 mM Mg2+ (o) or 1 µM Pb2+ (●), 50 mM imidazole acetate pH 7.0 and 0.1 mg/ml enzyme, dephosphorylation is performed in a medium which contains extra 0.5 mM non-radioactive ATP and K^+ as indicated. The % of E-P hydrolysed during 3 seconds incubation is plotted on the Y-axis.
Figure 3B. Effect of the Mg2+ concentration present during phosphorylation in addition to 3 µM Pb2+ on the K-sensitivity of the phosphoenzyme.
Figure 3C. Effect of the Na^+ concentration present during phosphorylation with Mg2+ (0.1 mM) and Pb2+ (3 µM) on the K-sensitivity of the phosphoenzyme.

Application of the Pb2+ activated phosphorylation in E1-E2 studies.

From the previous data we know that during phosphorylation in the presence of Pb2+ a phospho-enzyme is formed which is totally insensitive to K^+ and Mg2+. Only in the presence of Na^+ the K-sensitive phosphointermediate (E-P.Mg) is formed. As the E1 form (Karlish et al., 1978) of the enzyme can phosphorylate and the E2 not, the method used here enables us to study the effects of ligands, which normally promote dephosphorylation, on the E1-E2 equilibrium and on the rate of its conversion.

Without preincubation, the enzyme in 50 mM imidazole is in the E1 form (Schuurmans Stekhoven et al., 1985). Only at high K^+ concentrations there is a decrease in the phosphor-

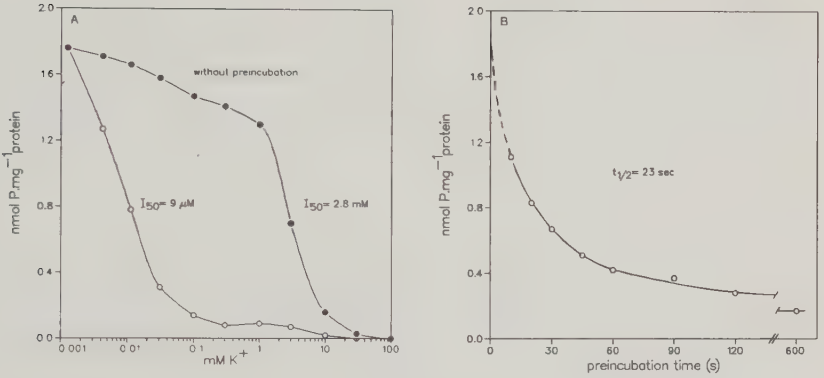

Figure 4A. Effect of K^+ on the E1/E2 ratio.
Phosphorylation is performed by 3 s incubation at 0 C with 5 µM [γ32P]ATP, 0.1 mM Mg2+, 3 µM Pb2+, 50 mM imidazole acetate, pH 7.0 and the indicated K^+ concentrations.
(●) Na,K-ATPase in 50 mM imidazole.
(o) Na,K-ATPase preincubated for 30 minutes at 0 C in 50 mM imidazole pH 7.0 and the indicated K^+ concentrations.
Figure 4B. Effect of 0.1 mM K^+ on the E1/E2 ratio of Na,K-ATPase in 50 mM imidazole at 0 C.

ylation level during the 3 second incubation period (Fig. 4A). The I-50 value for this K^+ effect is 2.8 mM. If Na,K-ATPase is preincubated in 50 mM imidazole with different concentrations of K^+ a new E1/E2 equilibrium is reached. The I-50 value for K^+ decreases 300 times to 9 µM. The latter value is also observed when studying the E1/E2 ratio with the extrinsic fluorescent probe 5'iodoacetamido-fluorescein (Fu et al., 1985).

Not only the ratio of E1/E2 can be studied with this technique but also the rate of the transition. The half time for the conversion from the E1 form (50 mM imidazole) to the E2 form with 100 µM K^+ is 23 seconds at 0 C (Fig. 4B).

CONCLUSIONS

1. Pb2+ increases in the absence of Na^+ the ATP phosphorylation level by increasing the phosphorylation rate constant, leading to a phosphoenzyme (E-P.Pb) which is insensitive to K^+.
2. Imidazole increases the steady-state phosphorylation by decreasing the dephosphorylation rate (reduction of the

K^+-sensitivity of E-P.Mg, Schuurmans Stekhoven et al., 1986).

3. Pb2+, additionally inhibits the steady-state phosphorylation, both in the presence and absence of Na^+. This inactivation is counteracted by Mg2+.
4. Via Pb2+ activated phosphorylation the E1/E2 transition can be studied, yielding a I-50 value for K^+ of 9 µM for the E1 form of Na,K-ATPase in 50 mM imidazole.

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STRUCTURAL CHANGES IN Na, K-ATPase ESTIMATED BY INTRINSIC AND EXTRINSIC FLUORESCENCE PROBES

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INTRODUCTION

Sequential appearance of E_1S^* (tight complex of Mg^{2+}, Na^+, ATP and the enzyme), E_1P (ADP-sensitive phosphoenzyme) E_2P (K^+-sensitive phosphoenzyme) and E_2 (dephosphoenzyme which can form E_2P from Pi) accompanied with conformational changes during Na, K-ATP hydrolysis has been shown clearly under steady and presteady state conditions by measurements of the changes in the fluorescence intensity and the amount of phosphoenzyme of N-[p-(2-benzimidazolyl)phenyl]maleimide (BIPM) modified Na, K-ATPase (Taniguchi et al., 1980, 1982, 1983, 1984, 1985, 1986, Suzuki et al., 1987). The sequential appearance of species of E_1P that precedes E_2P formation during the hydrolysis of ATP (Taniguchi et al., 1986) has also been shown by oligomycin sensitive dynamic light scattering changes. It was also shown that ATP did not bind to E_1P in such a way as to increase the extent of phosphorylation further or to accelerate dephosphorylation (Taniguchi et al., 1986) and that the three dimensional structure of Na, K-ATPase, which may be important in the dynamic fluorescence and light scattering changes, is little affected in spite of extensive covalent bond splitting in the α-chain (Inoue et al., 1987). Our data also showed that the conformational evidence for the change in the rate determining step in the presence of low and high concentrations of ATP during Na, K-ATPase reaction and that the role of high concentrations of ATP to accelerate the reaction can be replaced by ATP analogues such as ADP, adenylyl imidodiphosphate and adenylyl (β,γ-methylene)diphosphonate (Suzuki et al., 1987). Recently the BIPM residues involved in the fluorescence change was

shown to be attached to Cys-964 (Nagai et al., 1986) in the α-chain of Na, K-ATPase (Shull et al., 1985). Our results clearly indicates that the maximum fluorescence increase and decrease of the BIPM residues occured, respectively, accompanying the transition of E_1P to E_2P (Taniguchi et al., 1982) and KE_2 (K^+ bound enzyme) to E_1S^* (Taniguchi et al., 1984, 1985).

In this paper, steady and transient states fluorescence changes due to the accumulation of E_1S^*, E_1P, E_2P, KE_2 and OuabainE_2P (OuabE_2P) were compared with the fluorescence intensity of NaE_1 (Na^+ bound enzyme) in BIPM modified enzyme with further treatment or pretreatment of the other fluorescent reagent. The fluorescence change induced by oligomycin in the presence of Na^+ to form occluded Na^+ (Esmann and Skou, 1985) was also measured.

METERIALS AND METHODS

The BIPM enzyme was further treated with N-[7-dimethylamino 4-coumarinyl]maleimide (DACM) (Sakuraya et al., 1987) or fluorescein isothiocyanate (Karlish, 1980, Hegyvary and Jorgensen, 1981) or Na, K-ATPase preparations were sequentially treated with 5-iodoaceteamide fluorescein (Kapakos and Steinberg, 1986) and BIPM. The fluorescence measurements were performed as described previously (Taniguchi et al., 1982) and the relative fluorescence intensity of NaE_1 was taken as a standard. The enzymes were phosphorylated in the presence of Mg^{2+} from ATP with 2 M Na^+ and 16 mM Na^+, respectively, to accumulate E_1P and E_2P. The addition of K^+ to give 1.6 mM to E_2P immediately induced the accumulation of KE_2. The addition of ouabain to E_2P induced the accumulation of OuabE_2P. The addition of oligomycin to NaE_1 induced the occlusion of Na^+ (Esmann and Skou, 1985). When the enzyme preparations treated with FITC were used, ATP was replaced with acetyl phosphate to phosphorylate the enzyme (Beaugé et al., 1985, Rephaeli et al., 1986).

RESULTS AND DISCUSSION

The extrinsic and intrinsic fluorescence (Karlish and Yates, 1978; Jorgensen and Petersen, 1979; Taniguchi et al., 1982) intensities of each intermediate accumulated were measured. The following results were obtained as shown in Fig. 1.

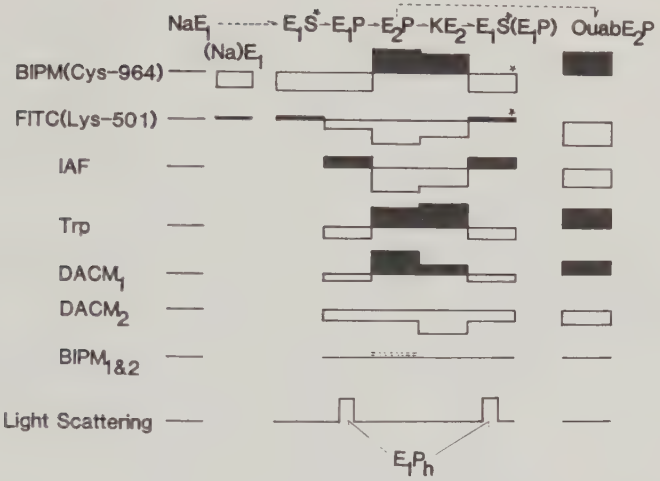

Fig.1. Changes in the fluorescence intensities during sequential appearence of intermediates in Na, K-ATPase. The reaction sequence is based on the Post-Albers mechanism (Albers, 1976, Post, 1979, Glynn, 1985).

1. The relative fluorescence intensities accompanying the formation of various enzyme species of the BIPM residue covalently bound to the sulfhydryl group of Cys-964 are as follows.

$E_1S^*=E_1P<(Na)E_1<NaE_1<KE_2<E_2P=OuabE_2P$

Where $(Na)E_1$ designates Na^+ occluded enzyme produced by oligomycin and NaE_1, — indicates the relative fluorescence intensity of NaE_1, ☐ and ■ indicate, respectively, the decrease and the increase in the fluorescence intensity.

2. Those of fluorescent isothiocyanate (FITC) residue cavalently bound to the ε-amino group of Lys-501 (Shull et al., 1985) in the BIPM treated enzyme are as follows.

$E_2P=OuabE_2P<KE_2<E_1P<NaE_1<(Na)E_1<E_1S^*$

3. Those of 5-iodoacetoamide fluorescein (IAF) residue are as follows.

$E_2P<KE_2=OuabE_2P<NaE_1<E_1P$

4. Those of tryptophan (Trp) residue in the BIPM treated enzyme are as follows.

$E_1P<NaE_1<E_2P=OuabE_2P<KE_2$

5. Those of N-[7-dimethylamino 4-coumarinyl]maleimide ($DACM_1$) residue present near some BIPM residue are as follows.

$E_1P<NaE_1<KE_2<OuabE_2P<E_2P$

6. Those of DACM residue ($DACM_2$) which does not accept fluorescence energy from the BIPM residue are as follows.

$KE_2 < E_1P = E_2P = OuabE_2P < NaE_1$

7. Two moles of BIPM residues present in the BIPM treated preparations not pretreated with NEM showed little fluorescence change accompanying formation of reaction intermediates. Only a slight increase in the fluorescence accompanying E_2P formation (===) was observed.

8. The increase in the light scattering intensity of BIPM modified enzyme was observed after the formation of E_1P. The intensity decreased before the formation of E_2P. The effect of oligomycin on the reversible light scattering changes and others suggested the sequential appearences of E_1P species (the subscripts $\ell(\ell')$ and h designate forms which have low and high light scattering intensity).

$E_1S^* \rightarrow E_1P\ell \rightarrow E_1P_h \rightarrow E_1P\ell' \rightarrow E_2P$

Where $\sqcap$ indicates the increase of the light scattering intensity.

The data clearly indicated that intrinsic and some extrinsic fluorescence probes sense occluded states for Na^+ namely $(Na)E_1$, E_1S^* and E_1P and for K^+, namely KE_2. Deocclusion of Na^+ and K^+ or decrease in the affinity for these cations, respectively to form E_2P from E_1P and E_1S^* from KE_2 seem to be simultaneous with the fluorescence changes. If the occlusion of ions increases the hydrophobicity in the vicinity of occluded ions the, data are compatible with the hypothesis that the decreases in FITC and IAF fluorescence intensities reflect the transition of occluded to deoccluded state for Na^+ ($E_1P \rightarrow E_2P$) and that the decrease in BIPM (Cys-964), Trp and $DACM_1$ fluorescence intensities reflect that for K^+($KE_2 \rightarrow E_1S$ or E_1P) : the increase in FITC and IAF fluorescence reflect K^+ deocclusion and Na^+ occlusion ($KE_2 \rightarrow E_1S^*$ or E_1P) and the increases in BIPM (Cys 964), Trp and $DACM_1$ reflect Na^+ deocclusion ($E_1P \rightarrow E_2P$) preceding K^+ occlusion (KE_2 formation).

These changes in different fluorescence residues suggest that two typical conformational changes occur accompanying the transition from E_1P to E_2P (reduction in the affinity for Na^+) and from KE_2 to E_1P via E_1S^* (reduction in the affinity for K^+). The magnitudes of these fluorescence changes were always larger than the other changes examined such as the transition from NaE_1 to $(Na)E_1$, NaE_1 to KE_2 and E_2P to KE_2.

Recently it was reported that Arg-262 and Arg-438 of α-chain in Na^+, K^+-ATPase were exposed in such a way as to be splitt by trypsin, respectively, in E_1 and E_2 forms of

protein (Jørgensen and Collins, 1986).

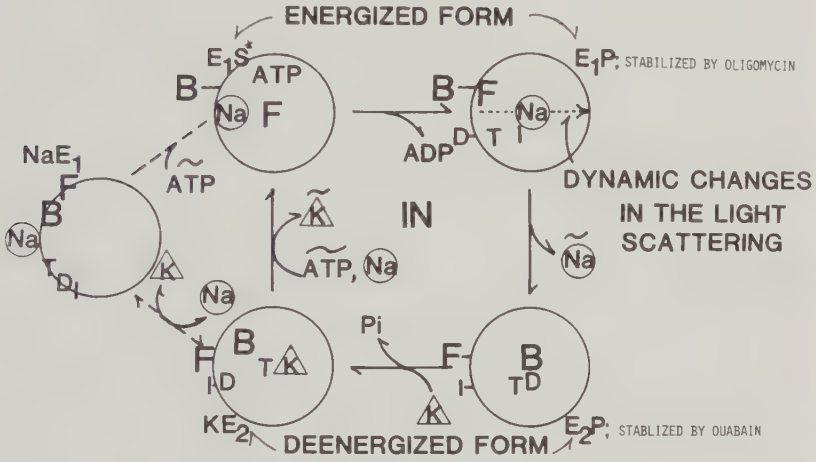

Fig.2. Conformational change during Na^+, K^+-ATPase reaction. Large circles represent different conformational states of Na, K-ATPase detected by phosphorylation kinetics and fluorescence measurements. The relative fluorescence intensities in NaE_1 of FITC(F), BIPM(B), Trp(T), $DACM_1$(D) and IAF(I) residues are taken as the standard. The location of these letters in and around large circles represent the relative fluorescence intensity : the inner the higher and the outer the lower. The small circle and triangle represent Na^+ and K^+, respectively. In this model we assume two major conformational states in the physiological condition, energized form (E_1S^* and E_1P) and deenergized form (E_2P and KE_2). Sodium ions bind cytosol side (left side of each circle) of the enzyme to form NaE_1. Occlusion of Na^+ requires the presence of Mg^{2+} and ATP(E_1S^*) or oligomycin.

Hypothetical model of conformational changes detected by intrinsic and extrinsic fluorescence and reversible light scattering intensity changes are shown (Fig. 2). In this model, free ATP is assumed to be in a high energy state (Boyer, 1977). Tight binding of ATP in the simultaneous presence of Mg^{2+} with Na^+ energized the enzyme to form E_1S^* (the free energy of ATP is trapped as a conformational energy) which conformational state permits to occlude Na^+.

Oligomycin may stabilize a conformational state which permits to occlude Na^+ without energy supply of free ATP. ATP splitting to form E_1P permitted to translocate the occluded Na^+ from the part of cytosol side in the enzyme to that of extracellular side in the enzyme. The process seems to be accompanied by the reversible light scattering changes. Oligomycin also stabilized the $E_1P(E_1P_h)$ which had high light scattering (Taniguchi et al., 1986).

Transition of a high energy E_1P to a low energy E_2P accompanied with the large reduction of the affinity (Taniguchi and Post, 1975) for Na^+ ($K_{0.5}$=0.6 M) with maximum increase or decrease in the extrinsic or intrinsic fluorescence intensities. Ouabain reduced the turnover of E_2P to form $OuabE_2P$.

Binding of K^+ to E_2P stimulated the liberation of Pi to form occluded K, namely KE_2 (Post, 1979, Glynn 1985).

Binding of ATP, Na^+ and Mg^{2+} reduced the affinity for K^+ to form a high energy E_1S^* with maximum decrease or increase in the extrinsic or intrinsic fluorescence intensities.

CONCLUSION

The data suggest that the free energy of ATP is transduced to the conformational energy in such a way as detected in the change of fluorescence intensities which occured out of phase during sequential appearence of reaction intermediates of Na, K-ATPase. The absence of fluorescence change in some BIPM residues suggests the presence of some domains with little change in the microenvironment during Na, K-ATP hydrolysis. It would be useful for better understanding of the mechanism of energy transduction to correlate both movements of Na^+ and K^+ in the molecule of Na, K-ATPase with sequential changes in these fluorescence intensities.

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QUENCHING OF THE TRYPTOPHAN FLUORESCENCE OF Na,K-ATPase WITH ACRYLAMIDE

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INTRODUCTION

The tryptophan fluorescence of Na,K-ATPase is increased by K; this increase is reversed by Na (Karlish and Yates, 1978). These changes, on the order of 1-2%, reflect transitions between the two major conformations of the enzyme, E_2 and E_1. In order to learn more about the basis for these fluorescence responses, quenching of the intrinsic (tryptophan) fluorescence by acrylamide was used to investigate the degree of exposure of the tryptophans in different conformations. Using the Stern-Volmer equation and Stern-Volmer plots modified to allow for heterogeneity in the tryptophans, the fraction of tryptophans most exposed to solvent (f_a), and their associated quenching constants (K_a) were calculated. In going from E_2 to E_1, a homogeneous population of accessible residues is converted into a heterogeneous one, along with a large increase in K_a. A population of completely inaccessible tryptophans persists under both conditions. Treatment with chymotrypsin to block the E_1 to E_2 transition produces a new set of quenching parameters that remain the same with Na or K. A more complete account of this work has appeared (Tyson and Steinberg, 1987).

METHODS

Na,K-ATPase was purified from frozen dog kidneys as described by Jorgensen (1974). Specific activity was

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between 10-18 μmol P_i formed per min per mg of protein. Fluorescence was measured on a Perkin-Elmer MPF-66 fluorescence spectrophotometer using a model 7500 computer. The sample compartment is equipped with a stirrer and temperature is maintained at 25°. Excitation was at 295 nm, and emission at 340 nm, with bandwidths of 5 nm. Fluorescence intensities are corrected for dilution and for absorbance of acrylamide itself using a molar extinction coefficient at 295 nm of 0.23 M^{-1} cm^{-1} (Parker, 1968). Chymotrypsin treatment was carried out according to Jorgensen and Petersen (1985).

Fluorescence quenching was analyzed using the Stern-Volmer equation ($F_o/F = 1 + K_a[Q]$) where F_o is the fluorescence in the absence of quencher, F is the fluorescence in the presence of quencher concentration [Q], and K_a is the Stern-Volmer quenching constant. Nonlinear Stern-Volmer plots can be generated by heterogeneity in the environments of the tryptophans in the protein; in this case, a modified Stern-Volmer equation ($F_o/F_o - F = 1/[Q]f_a K_a + 1/f_a$) is used (Lehrer and Leavis, 1978). Plotting $F_o/F_o - F$ vs $[Q]^{-1}$, the values for f_a, the fractional accessible fluorescence and K_a, the quenching constant, can be obtained. If the modified plots are curved, extrapolation from the lowest quencher concentration gives information about the most accessible residues. These are termed "effective" constants.

RESULTS AND DISCUSSION

Effect of Na and ADP on Quenching

Acrylamide quenches the fluorescence of Na,K-ATPase without shifting the wavelength of emission. Enzymatic activity is unaffected by acrylamide concentrations up to 300 mM, and only slightly inhibited at 500 mM.

With Na or ADP added to stabilize the E_1 form of the enzyme, the Stern-Volmer plots are quite similar and curve downward, indicating heterogeneity in the degree of exposure of the tryptophans to quencher. When replotted according to Lehrer and Leavis (1978) the modified plots are also nonlinear (Fig. 1A). Such behavior results from unquenched (buried) tryptophans, as well as unequal quenching of exposed residues, with the most accessible tryptophans being quenched at the lowest acrylamide

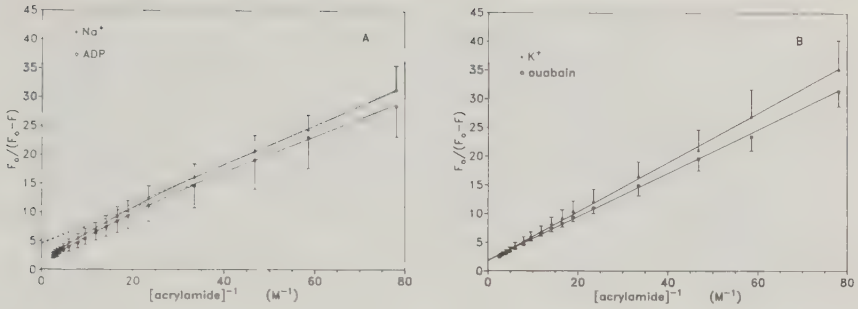

Figure 1. Modified Stern-Volmer plots in the presence of (A) 3 mM Na or 30 μM ADP and (B) 500 μM K or 0.1 mM ouabain +3 mM Mg. 0 to 400 mM acrylamide was added to 20 μg/ml enzyme in 100 mM Tris-HCl, 0.1 mM EDTA (pH 7.5) at 25°C. The data are plotted as described in Methods. (Courtesy J. Biol. Chem.)

concentrations. The K_a for these residues (with both Na and ADP) is ca. 14 M^{-1}, consistent with high accessibility, while the f_a is ca. 0.2, indicating a relatively small fraction of the total (Table 1).

TABLE 1. Quenching Parameters Determined from Modified Stern-Volmer Plots (Courtesy J. Biol. Chem.)

Additions	f_a	$K_a(M^{-1})$
Untreated Na,K-ATPase		
Na, 3 mM	0.22[1]	14.0[1]
ADP, 30 μM	0.22[1]	14.4[1]
K, 500 μM	0.57	4.1
Ouabain, 100 μM	0.53	4.9
α-Chymotrypsin treated		
Na, 3 mM	0.42[1]	11.0[1]
K, 500 μM	0.45[1]	11.1[1]

[1]Effective constants

Effect of K and Ouabain on Quenching

K and ouabain bind preferentially to E_2. Stern-Volmer plots with these ligands are nonlinear, as above, while the modified plots are linear (Fig. 1B). The lines for K and ouabain are very similar, with similar quenching parameters (Table 1). These values, f_a ca. 0.5 and K_a ca. 4 M^{-1}, would indicate that about half of the tryptophans are a homogeneous class of residues that are less accessible than in E_1.

Chymotrypsin Treatment

Jorgensen and Petersen (1985) showed that mild chymotrypsin treatment in the presence of Na cleaves a single bond in the alpha subunit, thereby trapping the enzyme in E_1. When chymotrypsin-treated enzyme is quenched with either Na or K present, the same results are obtained: curved modified Stern-Volmer plots with quenching parameters intermediate between E_1 & E_2 (Fig. 2 & Table 1)

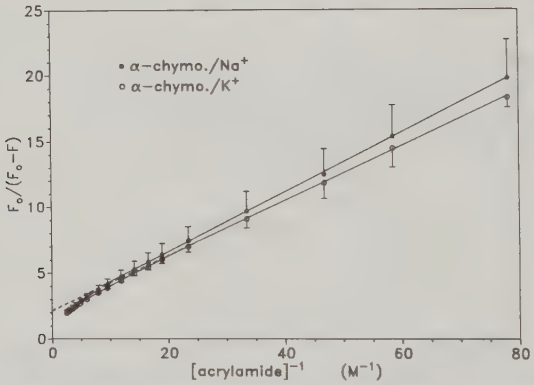

Figure 2. Modified Stern-Volmer plots of chymotrypsin-treated Na,K-ATPase. After limited proteolysis with chymotrypsin, either 3 mM Na or 500 μM K was added, and quenching recorded as in Figure 1. (Courtesy J. Biol. Chem.)

These results are consistent with chymotrypsin preventing the K induced change to E_2. However, it is clear that the treated enzyme is not identical to E_1. It is interesting to note in this regard that chymotrypsin-treated enzyme resembles E_2 in its ability to occlude K (Glynn et al., 1985), while it resembles E_1 is having a high affinity for nucleotides (Jorgensen, 1985).

CONCLUSIONS

The fluorescence quenching behavior of the tryptophans of Na,K-ATPase changes markedly when the enzyme converts from the E_1 to the E_2 conformation. In the E_1 state, stabilized by either Na^+ or ADP, the population of accessible tryptophans is small and heterogeneous, with a large K_a. In the E_2 state, stabilized with either K^+ or ouabain, the population of accessible tryptophans is larger and homogeneous, but less available to solvent. Chymotrypsin treatment results in an altered enzyme form, similar to E_1, that is unresponsive to K^+. These results provide new evidence that a significant rearrangement of amino acid residues results from the E_1 to E_2 transition. They also give some insight into the basis for the sensitivity of the tryptophan fluorescence to conformational state.

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OCCLUSION OF $^{22}Na^+$ AND $^{86}Rb^+$ IN MEMBRANE-BOUND AND SOLUBLE PROTOMERIC αβ-UNITS OF Na,K-ATPase

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INTRODUCTION

In the process of active Na,K-transport, the cations pass states, in which they are trapped in the structure of the pump protein, exchanging with medium cations only at a very low rate. Post et al. (1972) originally suggested the term "occlusion" for the state of Rb^+ in Na,K-ATPase after dephosphorylation. Beauge and Glynn (1979) were able to measure directly occlusion of K^+ or Rb^+ using Dowex columns for removal of freely exchangeable ions. Kinetic studies show that the Rb^+-occluded form of the protein is an energy well from which exit in either direction is slow (Karlish and Stein, 1982). ATP stimulates the rate of the conformational transition $E_2Rb - E_1Rb$, resulting in release of occluded Rb^+ ions at the cytoplasmic surface (Karlish and Stein, 1982; Karlish et al., 1982). Phosphorylation of the protein with P_i causes ordered release (Glynn et al., 1985) of occluded Rb^+ ions at the extracellular surface of the pump (Karlish et al.,1982). Thus, the direction of movement of the Rb^+ ions out of the occluded state is controlled by physiological ligands (Karlish et al., 1982).

Occlusion of Na^+ has been demonstrated after chymotryptic cleavage (Glynn et al., 1984) and after treatment with oligomycin (Esmann and Skou, 1985). Both procedures stabilize the Na,K-pump protein in the E_1P conformation in presence of ATP.

If a cavity for occlusion of cation is part of the pathway for transport of Na^+ and K^+ across the membrane, the minimum protein unit required for occlusion defines the unit that contains this cation pathway. Previously, it was shown that the soluble monomeric αβ-unit possesses Na,K-ATPase activity (Brotherus et

al.,1983; Craig, 1982), and that it can assume three (E_1Na, E_2K and E_2P) of the four major conformational states in the E_1-E_2 reaction cycle of the Na,K-pump (Jørgensen and Andersen, 1986). It was, however, not possible to stabilize the E_1P conformation in the soluble state, and thus determine if soluble αβ-units can assume the E_1P form or if this involves association between αβ-units (Periyasamy et al., 1983).

The purpose of this investigation has been to examine whether soluble monomeric αβ-units can occlude Rb^+ and Na^+ or if this requires formation of oligomeric $(\alpha\beta)_2$-units. For occlusion of $^{86}Rb^+$, we used established procedures (Glynn and Richards,1982). For occlusion of $^{22}Na^+$ we examined if formation of the phosphorylated complex of Na,K-ATPase from CrATP (Pauls et al., 1980) would cause occlusion of $^{22}Na^+$. The optimum conditions for occlusion of $^{22}Na^+$ in membrane-bound and soluble Na,K-ATPase were determined, and the sedimentation properties of the Rb^+-or Na^+-occluded phosphorylated complexes were studied in the analytical ultracentrifuge. The stoichiometry for cation occlusion was measured relative to ^{32}P incorporation from [γ^{32}-P]CrATP and binding of [^{48}V]vanadate and [^3H]ouabain (Vilsen et al. 1987).

RESULTS AND DISCUSSION

Na^+ Occlusion

Reaction of Na,K-ATPase with CrATP leads to occlusion of Na^+ ions. Fig. 1A shows the amount of occluded Na^+ as a function of Na^+ concentration for membrane-bound Na,K-ATPase and soluble Na,K-ATPase. Optimum conditions for occlusion of Na^+ were found to be 3 hours incubation with 0.5 mM CrATP in presence of 1 mM Mg^{2+} and absence of K^+. Thus, Na^+-occlusion is a slow process in accordance with previous data demonstrating a slow rate of phosphorylation of Na,K-ATPase by CrATP (Pauls et al., 1980). Addition of K^+ together with Na^+ in the incubation medium decreases the steady-state level of Na^+ occlusion, presumeably by binding to specific cation sites (Matsui and Homareda, 1982)

The data obtained with membranous Na,K-ATPase have been fitted to Hill's equation, (Fig.1A) discarding the points corresponding to Na^+ concentrations above 2 mM, where the determinations are uncertain . The fit results in a $K_{\frac{1}{2}}$ of 0.95 mM, a maximum site concentration of 12.9 nmol of occluded Na^+/mg of protein, and a Hill number of 1.5.

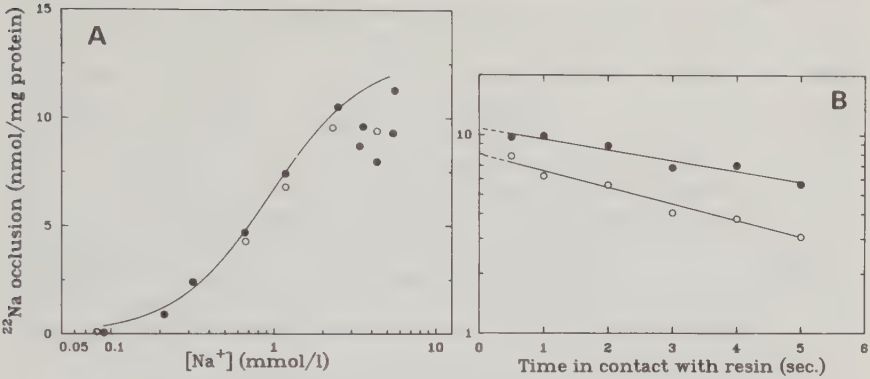

Figure 1. CrATP induced Na^+ occlusion in membrane-bound (●) and soluble Na,K-ATPase (O). (A) Na^+ concentration dependence. The Na^+ occluded complex was formed by incubation of purified Na,K-ATPase in membrane-bound form with $^{22}Na^+$ and CrATP for 3 hours. Solubilization with $C_{12}E_8$ (3 mg/mg protein) was performed 5 min. prior to termination of the incubation.
(B) Release of occluded Na^+.

In Fig.1B, we have determined the rate of release of occluded Na^+ from membrane-bound and soluble Na,K-ATPase by varying the rate of passage through the Dowex column. The loss of occluded Na^+ has an exponential time course in both preparations and the occluded ions are released from the soluble enzyme at a slow rate. This is similar to the rate observed in membranous Na,K-ATPase and comparable to the rate measured by Glynn et al. (1984) after modification with chymotrypsin. In Fig.1B we have used preparations with particularly high specific activities of Na,K-ATPase (46.3 µmol/mg/min) in order to determine the maximum level of Na^+ occlusion. Extrapolation of the lines to zero time permits correction for loss of Na^+ during the passage down the Dowex column. This results in maximum values of 10.8 and 8 nmol Na^+ occluded/mg protein for membrane-bound and soluble Na,K-ATPase, respectively. Previously, evidence of occlusion of Na^+ has been incomplete due to difficulties involved in isolation of the ADP-sensitive phosphoenzyme. Maximum values of only 2-3 nmol of occluded Na^+/mg of protein have been measured on enzyme pretreated with either α-chymotrypsin (Glynn et al., 1984) or with N-ethylmaleimide (Glynn et al., 1984). Using CrATP as phosphorylating substrate, thus enabled us to measure 3-4 times

higher values of Na^+ occlusion. With soluble enzyme we obtained 10-25 % lower maximum values of Na^+ occlusion than with membrane-bound Na,K-ATPase. Studies of Na,K-ATPase activity have shown that this is due to irreversible inactivation occuring upon solubilization. We found that maximum levels of occlusion were obtained if the occluded complex was formed in the membrane-bound state, before solubilization in $C_{12}E_8$. When the order of the experiment was reversed so that Na,K-ATPase was rendered soluble before reaction with $^{22}Na^+$, relatively low levels of occlusion were obtained. By examination of the stability of soluble enzyme without and with specific cations (Na^+,K^+ and Rb^+) in the medium, this inactivation was found to result from instability of the unliganded ("empty") soluble Na,K-ATPase.

Determination of Stoichiometry

This requires at least three corrections of the measured values of Na^+ occlusion. The first correction is for the loss of Na^+ that occurs during the passage of the enzyme down the ion exchange column (see above). The second correction is for lack of saturation of Na^+-binding sites (Fig. 1A). This is performed by extrapolation from values of Na^+ occlusion measured at low Na^+ concentration to the amount occluded at saturating concentrations assuming the dependence of occlusion of Na^+ concentration indicated by the line in Fig.1A. The third correction is for variation of enzyme purity as indicated by the specific Na,K-ATPase activity. Table 1 shows specific activities of a series of selected Na,K-ATPase preparations and corresponding data of phosphorylation from [γ-^{32}P]CrATP. As seen from the table a high specific activity of 46.5 µmol/min/mg of protein is matched by a high level of phosphorylation of 6.0 nmol of EP/mg of protein, whereas a relatively low specific activity of 31.1 µmol/min/mg of protein corresponds to a phosphorylation level of only 3.9 nmol of EP/mg of protein. The measured EP levels in the soluble enzyme are somewhat lower than in membrane-bound Na,K-ATPase. Our data yield stoichiometries ranging from 1.8 to 2.7 mol of Na^+/mol of EP. The latter value is obtained after the three corrections of the measured values mentioned above. The value of 1.8 is a lower limit calculated directly from the measured numbers. There was no significant difference between the stoichiometries obtained with soluble enzyme and membrane-bound Na,K-ATPase. On the basis of measured values for Na^+ occlusion and EP level which were 5-10 fold lower than those determined in the present study, Glynn et al. (1984) calculated a stoichiometry of 2.8 mol of occluded Na^+/mol of EP in chymotrypsin-cleaved enzyme. This result, as well as ours,

agrees reasonably well with the accepted transport stoichiometry of 3 Na^+ ions transported per molecule of ATP hydrolyzed (Glynn and Karlish, 1975).

Table 1.

Relation between specific activity of Na,K-ATPase and capacity for phosphorylation from [γ-^{32}P]CrATP.

Na,K-ATPase activity μmol P_i/mg protein/min	Phosphorylation from [γ-^{32}P]CrATP nmol/mg protein
Membrane bound Na,K-ATPase	
46.5	6.0
42.0	5.5
39.0	4.4
36.0	5.0
31.1	3.9
Soluble Na,K-ATPase	
41.0	4.5
39.0	3.6

Occlusion of Rb^+

Experiments were performed to investigate if soluble Na,K-ATPase can occlude Rb^+ ions to the same extent as membrane-bound Na,K-ATPase. The relation between the amount of Rb^+ occluded after passage of the enzyme down the ion exchange column and the Rb^+ concentration is shown in Fig. 2A for membrane-bound as well as for soluble Na,K-ATPase. We have not studied occlusion of Rb^+ at concentrations below 50 μM because the soluble Na,K-ATPase is unstable at low cation concentrations (see above). Also shown in Fig. 2A are data representing total Rb^+ binding (▲) at equilibrium i.e. including freely exchangeable Rb^+. It is obvious that there is no significant difference between the levels of binding and occlusion, indicating that the sites involved in Rb^+ occlusion are not distinct from those of Rb^+ binding. Fig. 2B shows that the rate of deocclusion of Rb^+ is extremely slow ($k<0.030\ s^{-1}$) for both membrane-bound and soluble enzyme. The extrapolated initial values are 10.3 and 8.0 nmol of occluded Rb^+/mg of protein for membrane-bound and soluble enzyme, respectively (specific activity 46.3 μmol/mg/min; like enzyme used in Fig.1B). Using the

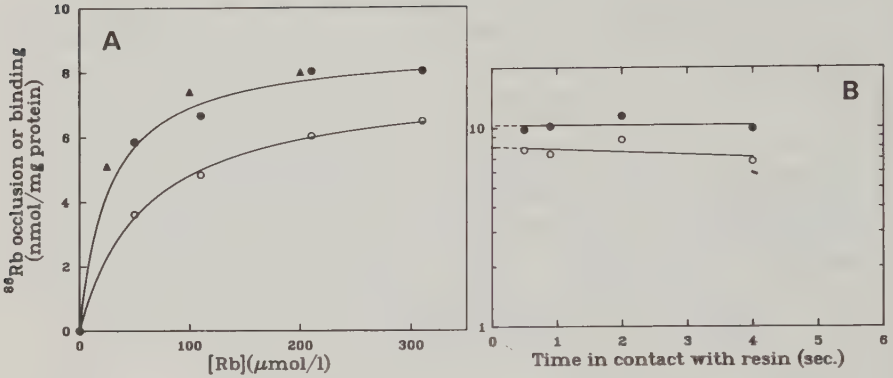

Figure 2. Rb^+ occlusion in membrane-bound (●,▲) and soluble (○) Na,K-ATPase. (A) Rb^+ concentration dependence. The Rb^+ occluded complex was formed by incubation purified Na,K-ATPase in membrane-bound form with $^{86}Rb^+$ for 15 min. Solubilization with $C_{12}E_8$ was performed as described in the legend to Fig.1A. (B) Release of occluded Rb^+.

site concentrations determined with [γ-^{32}P]CrATP and extrapolation to saturating concentrations of Rb^+ (cf. Fig.2A) as discussed for Na^+ occlusion above, we obtain a stoichiometry of 2.0 and 2.4 mol of Rb^+/mol of enzyme for membranous Na,K-ATPase and soluble enzyme, respectively. Our results on Rb^+ occlusion indicate that membrane-bound as well as soluble Na,K-ATPase in the dephosphoform is capable of occluding 2 mol of Rb^+/mol of active site. This agrees with the transport stoichiometry of 2 Rb^+ or K^+ ions transferred across the membrane in each transport cycle (Glynn and Karlish, 1975; Sen and Post, 1964). In contrast Glynn and Richards (1982) have found a stoichiometry of 3 Rb^+ ions occluded per phosphorylation site for membrane-bound enzyme.

Self-association state of soluble Na,K-ATPase

In the analytical ultracentrifuge, the soluble Na,K-ATPase in the phosphorylated Na^+ occluded form as well as in the Rb^+ occluded form was found to have a sedimentation coefficient ranging between 6.8 and 7.4 S. It was found to be independent of the duration of incubation with CrATP. As previously documented, this range of sedimentation coefficients are consistent with the protein existing predominantly as αβ-units with molecular weights in the range 143,000-170,000 (Jørgensen and Andersen, 1986).

An αβ protomer as the minimum functional unit is also supported by the stoichiometry of 6.0 nmol EP/mg protein measured in preparations of high specific activity. We have measured similar values (5.3-6.4 nmol/mg) for binding of ouabain and vanadate. Thus our data show that under optimum conditions each mole of αβ-unit binds 1 mol of vanadate or ouabain and incorporates 1 mol phosphate from [γ-^{32}P]CrATP.

Concluding Remarks

The αβ-unit can assume the dephosphoforms E_1Na and E_2K (Jørgensen and Andersen, 1986), and this paper shows that it occludes 2 Rb^+ ions. The soluble monomeric αβ-unit also assumes the phosphorylated E_2P form (Jørgensen and Andersen, 1986), and the present data demonstrate that both in membranous and soluble monomeric Na,K-ATPase, phosphorylation from CrATP stabilizes a Na^+occluded conformation reminiscent of the E_1P form. Thus, it is possible to conclude that the soluble αβ-unit can assume all of the four major conformation that Na,K-ATPase must pass to carry out ion translocation in the E_1-E_2 reaction cycle. This together with the demonstration that soluble αβ-units of the Na,K-ATPase occlude Na^+ and Rb^+ with the same stoichiometry and kinetics as membrane-bound enzyme indicate that occlusion is not due to segregation of the ions in a channel-like compartment formed between subunits in an $(\alpha\beta)_2$-dimer, neither is it due to movement of ion-binding sites into the bilayer structure, since the latter has been broken down by formation of mixed micelles of lipid and detergent in the solubilization process. The present data suggest that the ions are occluded in a presumptive "cavity" that is formed within the structure of the αβ-unit.

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EFFECT OF CYTOPLASMIC K^+ ON ADP- AND K^+-SENSITIVE EP OF RECONSTITUTED NaK-ATPase PROTEOLIPOSOMES

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INTRODUCTION

The two phosphorylated forms of NaK-ATPase (EP) - an ADP-sensitive K^+-insensitive EP (E_1P) and a K^+-sensitive ADP-insensitive EP (E_2P) - are the reaction intermediates of the ATP-hydrolysis in the absence of K^+. Recently, it has been shown that the sum of the ADP- and K^+-sensitive EP's exceeds the amount of total EP in certain enzyme preparations. This result implies the existence of a third EP, the intermediate of the E_1P to E_2P conversion - the ADP- and K^+-sensitive E*P. In the fragmental NaK-ATPase, the EP composition varies with the Na^+ concentration, even though the total EP level remains constant (Nørby et al., 1983; Yoda and Yoda, 1986). In the reconstituted NaK-ATPase proteoliposomes (PL), however, the EP composition of the inside-out form is influenced not only by the Na^+ concentration in the media but also by the temperature and cholesterol content of the lipid bilayer. These results suggest that the barrier effects of the closed lipid bilayer control the conversion of E_1P to E_2P via E*P. In addition to the acceleration of its dephosphorylation by both ADP and K^+, the E*P is thought to be the high-affinity form of NaK-ATPase for the cardiac steroid binding (Yoda and Yoda, 1987a).

The above results were obtained without K^+. By using PL, we extended these studies to E*P in the presence of K^+ and found that the cytoplasmic

(extravesicular) K^+ (K^+_{cyt}) affects the change of E*P to E_2P.

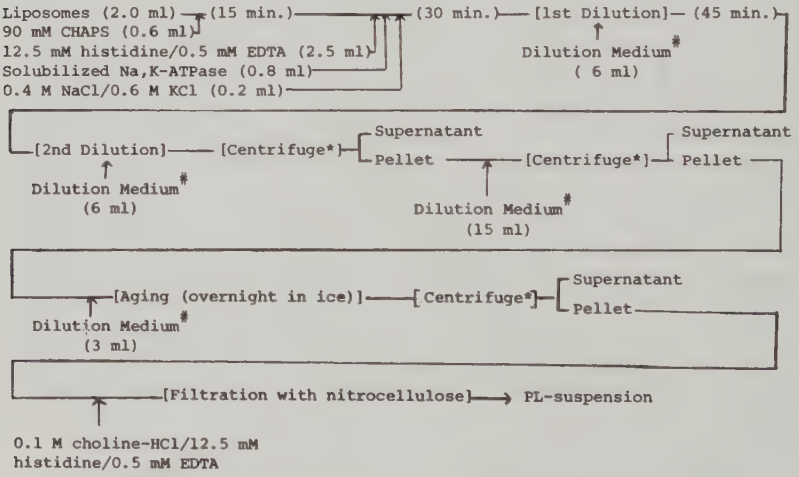
METHODS

The fragmental NaK-ATPase used in this study was prepared from the electric organ of Electrophorus electricus and was reconstituted into the cholesterol-containing egg phospholipid liposomes after solubilizing with cholamidopropyl dimethylammonio propane-sulfonate (CHAPS). This reconstitution method was modified as shown in Fig. 1. The main change from the previous method

Liposomes, The mixture of egg phospholipid (50 mg/ml) and cholesterol (15 mg/ml) was sonicated.

Solubilization of Na,K-ATPase, The mixture containing fragmental Na,K-ATPase (1.2 mg/ml), 0.5 M NaCl, 9 mM CHAPS. 12.5 mM histidine, and 0.5 mM EDTA (pH 7.0) was incubated for 30 min. at 15°, and the non-solubilized residue was removed by centrifugation (540,000 xg. 15 min.).

Reconstitution, (for the PL's which intravesicular medium was 80 mM NaCl/20 mM KCl/12.5 mM histidine/0.5 mM EDTA) All procedures were performed at 15° except the aging.



same as the intravesicular medium.

* 540,000 xg, 25 min. [Beckman Tl-100 centrifuge and Tl 100-3 rotor]

Figure 1. Improved reconstitution procedure.

(Yoda et al., 1984) was the use of centrifugation instead of gel filtration to remove the CHAPS. This improvement avoided the troubles caused by the absorption of lipids on the gel (Sephadex G25). The PL used here contained 100 mM NaCl or 80 mM NaCl plus 20 mM KCl as the salt in the intravesicular (extra- cellular) medium.

The determination of the EP composition was performed at 15°C. After 2 sec of the reaction of the PL in a mixture of 20 mM NaCl/5 mM free $MgCl_2$/10 μM ^{32}P-ATP/25 mM histidine buffer (pH 7.4) with or without 20 mM KCl, the phosphorylation was quenched and the dephosphorylation was started with 20 mM $Tris_3$ CDTA with or without 0.2 mM ADP and/or 2 mM KCl plus 20 μM nigericin. The total salt concentration in these reaction media was kept at 100 mM with choline HCl. After 0.4 to 3 sec of dephosphorylation, the PL were denatured with TCA and the amount of EP remaining was measured. The dephosphoryla- tion curve with ADP or K^+ plus nigercin was biphasic (see Fig. 2). The amount and the dephosphorylation rate constant (k_d) of ADP-insensitive EP (E_2P) or K^+-insensitive EP (E_1P) were estimated from the decay curve of the slower phase, and the E*P content was calculated from the difference between the total EP content and the sum of the E_1P and E_2P amounts.

The ATPase activity of the PL was measured by the formation of ^{32}P-inorganic phosphate from 0.1 mM ^{32}P-ATP in the presence of 20 mM NaCl/0.3 mM ouabain/5 mM $MgCl_2$ with or without 20 mM KCl for 20 or 40 sec at 15°C, as reported previously (Yoda et al., 1984). The total salt concentration in the reaction medium was kept at 100 mM with triethanolamine HCl. The assay medium without NaCl was used as the control.

The comparison of the amounts of the inside-out form in the various PL was based on the maximum NaK-ATPase activity, which was the ATPase activity at 30°C in the presence of 100 mM NaCl/20 mM KCl/1 mM $MgCl_2$/1 mM ATP 0.3 mM oubain/20 μM monensin/20 μM nigericin. Under this condition, we assumed that the concentrations of Na^+ and K^+ on both sides of the PL were kept at 100 mM Na^+ and 20 mM K^+ during the reaction period (1 to 10 min) and also that the PL remained intact in the presence of both ionophores during this interval.

RESULTS AND DISCUSSION

The EP level in the fragmental NaK-ATPase decreased to about 40% of the original value in the presence of 5 mM K^+. A significant decrease in the EP level was observed in the reconstituted PL only when K^+ was present in the extracellular medium. If the EP levels were calculated using the same value for the maximum ATPase activity, the level with 20 mM extracellular K^+ (K^+_{ex}) was 63 $\pm$ 3% of that for the PL without K^+ on both sides. However, if K^+ was present only on the cytoplasmic side of the PL, no significant decrease in the EP level was found.

The k_d's of E_1P were not affected by 20 mM K^+_{cyt}, as shown in Table 1. Therefore, K^+_{cyt} probably did not change the properties of E_1P and E_2P.

Since the E*P and E_2P are K^+-sensitive, the EP of the PL formed with 20 mM K^+ on both sides was expected to be only the K^+-insensitive E_1P. However, although the EP obtained was sensitive to ADP, the dephosphorylation curve was still biphasic (closed circle, solid line in Fig. 2). Based on the k_d's shown in Table 1, the component of the EP with the slower dephosphorylation was assumed to be E_1P; it was calculated from extrapolation of the dephosphorylation curve to be about 50% of the total EP.

Since E*P reacts rapidly with digitoxigenin to form the ADP- and K^+-insensitive EP, digitoxigenin was used to determine whether the component of this EP with the faster dephosphorylation was E*P. As shown by the dashed line in Fig. 2, the addition of digitoxigenin (final concentration 0.1 mM) with the quenching of phosphorylation changed 47% of the EP to the ADP- and K^+-insensitive form with a k_d of 0.21 sec^{-1} at 15°C. The amount of the obtained insensitive EP was almost equal to that of the rapidly dephosphorylating EP described above. Therefore, we concluded that the PL formed a mixture of E_1P and E*P in the presence of K^+ on both sides. We also concluded that the dephosphorylation of E*P by K^+ (even with K^+ on both sides) was slower than the formation of E*P and the dephosphorylation of E_2P by K^+_{ex}.

TABLE 1. k_d of EP at 15°C

Salt during phosphorylation* $[K^+_{cyt}]$	$[K^+_{ex}]$	$[Na^+_{ex}]$	Additive in quencher (CDTA)	Type of EP**	k_d
mM	mM	mM			sec^{-1}
0	0	100	ADP	E_2P	-0.88
20	0	100	ADP	E_2P	-0.99
0	0	100	K^+, nigericin	E_1P	-0.31
20	0	100	K^+, nigericin	E_1P	-0.30
0	20	80	K^+, nigericin	E_1P	-0.33
20	20	80	$\pm$ nigericin	E_1P	-0.34

* $[Na^+_{cyt}]$ = 20 mM in all experiments.
** EP with the slower dephosphorylation.

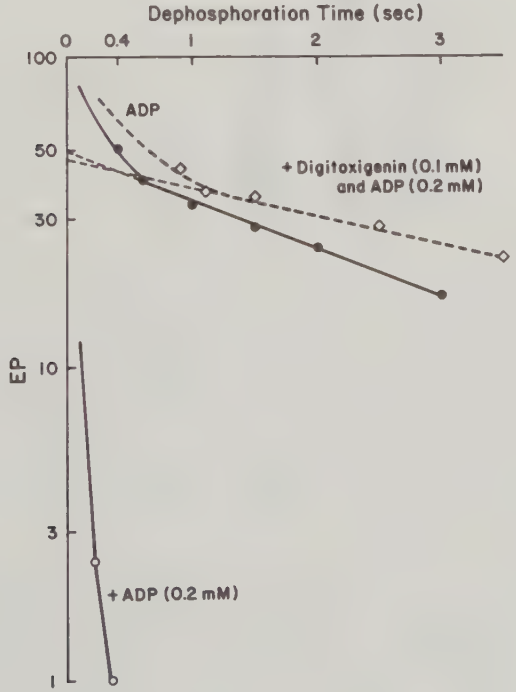


Figure 2. Dephosphorylation curve of NaK-ATPase proteoliposomes in the presence of 20 mM K^+ on both sides.

The effects of Na^+ and K^+ on both sides of the PL on the EP level and its composition are summarized in Fig. 3. In the absence of K^+_{ex}, the presence of K^+_{cyt} did not change the total EP level, but it did increase the E_2P content. Although the amount of E_2P was decreased by K^+_{ex}, E*P was not rapidly dephosphorylated by K^+_{ex}. These results suggest that E*P reacts with the K^+_{cyt} and changes to E_2P but does not react directly with K^+_{ex}. This conclusion indicates that the sidedness effects of K^+ on E*P are opposite to those on E_2P.

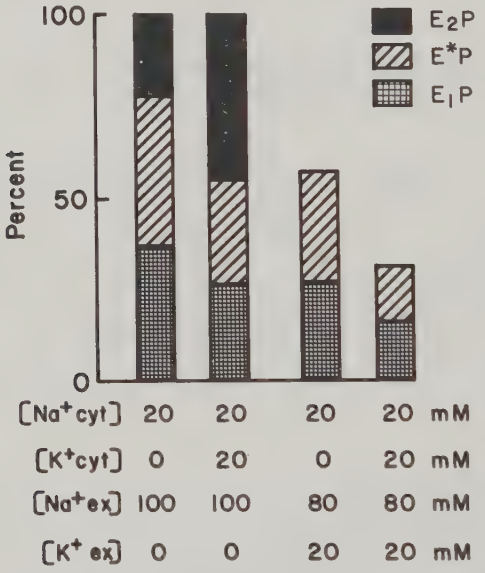


Figure 3. Change of EP level and composition by K^+ in both media.

If the above conclusion is correct, K^+_{cyt} may enhance the ATP-hydrolysis in the PL when the E_1P to E_2P conversion controls its enzyme turnover. As shown in Fig. 4, the ATPase activity of the PL was apparently enhanced by 20 mM K^+_{cyt}. This enchancement by K^+_{cyt} was not observed when K^+_{ex} was absent (data

not shown). [Our preliminary results also suggest that K^+_{cyt} enhances the ^{22}Na-uptake in the PL.]

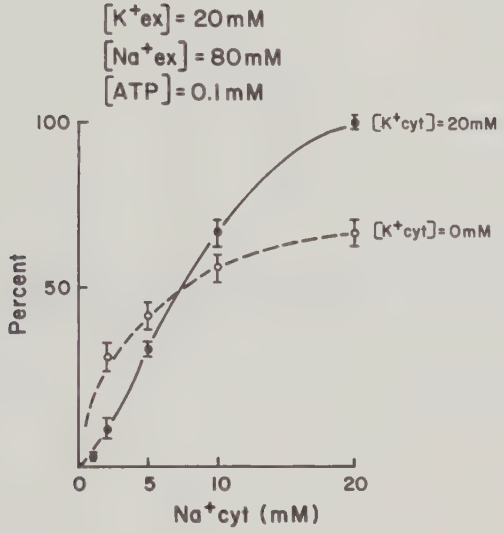


Figure 4. Effect of K^+_{cyt} on ATPase activity.

This study using reconstituted NaK-ATPase proteoliposomes shows that K^+_{cyt} interacts with E*P to accelerate conversion to E_2P, but a direct interaction between E*P and K^+_{ex} is unlikely. Consequently, the ATP-hydrolysis of the NaK-ATPase proteoliposomes is enhanced when K^+ is present on both sides. Since E*P has been previously postulated to bind with two Na^+ ions (Yoda and Yoda, 1987b), these results suggest the simultaneous binding of Na^+ and K^+ on the E*P form.

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Transport Mechanism: Molecular Aspects

The Na^+,K^+-Pump, Part A: Molecular Aspects, pages 401-420
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OVERVIEW:
MECHANISM OF FREE ENERGY COUPLING BETWEEN ATP HYDROLYSIS AND ION TRANSPORT

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INTRODUCTION

A lot of things are true without being useful: for example ion transport equations derived from irreversible thermodynamics. This paper will present a theoretical model for ion transport that focuses on the protein that is doing the transporting.

The theory described is at present incomplete, and this paper in particular is limited to consideration of three very basic questions. For the participants in this conference, there is an additional limitation - I shall consider these basic questions with reference to the sarcoplasmic reticulum (SR) Ca^{2+} pump and not with reference to the Na^+,K^+ system. However, that may be less of a disadvantage than might at first appear. The two pump molecules are known to be broadly similar, and it is very likely that there are substantial similarities in their transport mechnaisms. Possible differences will be mentioned in the Discussion.

More important, the questions that need to be asked are the same for the two pumps - note that the name of the transported ion(s) does not appear in the title of the paper. And at the present stage of our knowledge of the mechanism, a statement made long ago by James Thurber is probably appropriate (Thurber, 1939):

"It is better to know some of the questions
than all of the answers."

The principal objective of the questions will be to try to relate the transport function to the structure of the transport proteins. When we started out, the available structural information was minimal: the ATP binding part of the protein was known to be on a large domain extending far into the aqueous medium on the cytoplasmic side of the membrane (which is where the ATP is). The ion translocating part had not really been identified experimentally as being anywhere in particular, but for obvious reasons it has to be in the domain of the lipid bilayer. More structural information has become available during the last year, and, as will be seen, it has proved very helpful. It has not contradicted the initial assignment of the ATP processing and ion translocating regions.

In line with the Thurber philosophy, we chose at first to ignore the problem of free energy coupling per se, the question of how the ATP processing and ion translocating regions communicate and interact. There were more pressing questions that first needed to be asked about ion translocation per se, questions which no-one had thought about much before:

(1) The transported ions (whether Ca^{2+} or Na^{+} or K^{+}) not only go from one side of the membrane to the other (there are lots of theories about that), but they also undergo a dramatic change in concentration: for example, from 10^{-7} M to above millimolar when Ca^{2+} goes from the muscle cell cytoplasm to the sarcoplasmic reticulum lumen. How can a protein catalyze a change in concentration? We know a lot about how enzymes work, but usually enzymes catalyze chemical transformations, by breaking certain chemical bonds in organic molecules and making new bonds to take their place. Here there is no chemical transformation, the Ca^{2+} ions are unaltered, only their concentration changes. How can a protein manage to catalyze something like that? We are dealing with a task outside of the normal biochemical experience.

And it should be noted that this question is independent of the coupling part of the problem. It applies equally, for example, to Na^{+}/Ca^{2+} exchange, where the free energy to drive Ca^{2+} uphill is derived from downhill transport of Na^{+} and not from ATP hydrolysis.

(2) The second question has to do with stoichiometry. The chemical reactions for pump proteins are always

written with specified transport stoichiometries: 2 Ca^{2+} per ATP for the SR Ca^{2+} pump, $Na^+/K^+/ATP = 3:2:1$ for Na^+, K^+ pumps. Are the stoichiometries really fixed, as the equations imply? If so, how is it done? How does a protein manage to act as a measuring device, so that it handles precisely 2 Ca^{2+} ions or 3 Na^+ ions in each cycle of events leading to translocation and the concomitant concentration change?

Again, this is a question that has nothing to do with ATP hydrolysis, for the Na^+/Ca^{2+} exchange protein is also generally assumed to work with constant stoichiometry: 3 Na^+ going one way for each Ca^{2+} going the other way.

Only after these initial questions were resolved (at the theoretical level) did we intend to approach the subject of the title of this paper, i.e., the question of interaction between ion translocation and ATP hydrolysis.

CHEMICAL POTENTIAL OF PROTEIN-BOUND IONS

The best way to approach the first question is to rephrase it in terms of chemical potentials, i.e., to replace the concentration difference across the membrane, C(in) versus C(out), by the corresponding difference in the chemical potential,

$$\Delta\mu = RT \ln C(out)/C(in) \qquad (1)$$

When dealing with the sarcoplasmic reticulum membrane, where there is no electrical potential difference across the membrane, we don't have to worry about whether the pure chemical potential as given by this equation is the appropriate thermodynamic parameter here or whether one should use the electrochemical potential instead, which includes the work done against an electrical field in addition to the osmotic (concentration) term. With the concentration gradient applicable to the SR pump, equation 1 yields a value of about 6 kcal/mole for $\Delta\mu$ per transported Ca^{2+} ion.

Instead of trying to visualize catalysis of a concentration change, the question now becomes "How does a protein manage to change the chemical potential of an ion?", and an obvious extension is "How can the chemical

potential be changed while an ion is bound to the protein?" That was an essential assumption, where our approach perhaps differed most strongly from other physico-chemical analyses. The protein is the catalyst, it doesn't act on ions far away in the solution, it must exert its influence on the ion while the ion is bound to the protein. And in the protein-bound state there is no concentration dependent term in the chemical potential; μ in equation 1 becomes equal to the standard potential μ^o, which in turn is related to the equilibrium constant for binding the ion to its binding site on the protein. In the case of Ca^{2+}, protein binding sites (and sites on non-protein model compounds) usually involve negatively charged COO^- groups and, to a lesser extent, other oxygen ligands. The strength of binding is largely determined by the number of chelating groups in the Ca^{2+} ion's coordination sphere - tight binding typically involves six chelating groups in an octahedral complex, weaker binding requires only 3 or 4 chelating groups (Tanford, 1982).

TABLE 1. Related Statements About the Chemical Potential of a Protein-bound Ligand.

Low potential	High potential
Low escaping tendency*	High escaping tendency
Tightly bound	Weakly bound
High Keq for binding	Low Keq for binding
Many chelating groups	Few chelating groups

*"Escaping tendency" is a very descriptive term that used to be popular in the teaching of thermodynamics (Lewis and Randall, 1923), but is not used much any more.

Table 1 summarizes these principles and shows how to think one's way from a change in chemical potential of a protein-bound ion to a change in protein structure. If one desires a picture, one might look at the Ca^{2+} binding site in one of the "EF hand" type of Ca^{2+} binding proteins. Parvalbumin, for example, has a very strong binding affinity: the binding site contains 4 COO^- groups, one carbonyl oxygen and one OH group (Kretsinger, 1976). Two of the COO^- groups derive from aspartyl and glutamyl residues on

a segment of protein that could (in a model) be twisted away from the binding region, and that would change the binding affinity from strong to weak.

That is then the sort of thing we need to look for as a central feature of the mechanism for changing the chemical potential - a change in the coordination sphere of the bound ion, while it still remains bound to the protein. Note that if we think about the problem in this way, then we essentially visualize the role of the transport protein as doing what all proteins (enzymes) are comfortable doing, making and breaking of bonds. Bonds are made to bind Ca^{2+} initially, some are broken to change the potential, the remaining ones are broken to release the Ca^{2+}. The bonds in this case are chelation bonds and not covalent bonds, but that can hardly be an important distinction from the protein's point of view.

Tanslocation Pathway

We have so far discussed the question of how to change the chemical potential, but have not yet related that to transport from one side of the membrane to the other. To do that we must have a mental picture of the translocation pathway, and this is a familiar subject. It has been understood for a long time that the translocation pathway for active transport can never be a channel that is simultaneously open to both sides of the membrane. Such a channel would always cause ions to flow downhill from high potential to low potential - no matter how one regulates the opening and closing of the channel or couples it to ATP hydrolysis. The next simplest possibility is then to have the protein shuttle between two conformational states, one open to one side of the membrane, the second state open to the other side (Tanford, 1983).

In other words, the minimal model for a translocation pathway, which has been called the "alternating access" model, involves two distinct conformational states. Combining this with the idea of a change in chemical potential as specified in Table 1 essentially dictates the thermodynamic properties of the two states. One, the uptake state, must have a high binding affinity for the transported ion, the other, the discharge state, must have low binding affinity.

Table 1 suggests that a change in binding affinity should correspond at the molecular level to a change in the number of ligating groups. The overall picture (at this stage purely speculative) might then be the "rocking helix" model I suggested some years ago (Tanford, 1982). The trans-membrane portion of the protein is visualized as a bundle of more or less parallel helices, but slanted so that access to one side of the membrane is open, whereas access to the other side is blocked. The direction of access in such a model is readily changed by means of a rocking motion of the helices. Adding a little twist could at the same time pull some ligating groups away from the bound ion to change the binding affinity.

A thermodynamic aspect of this kind of model is that the translocation step must then also be the locus of energetic coupling. It takes just as much energy to pull ligating groups away from a bound ligand as it does to pull a ligand away from its binding site. The postulated rocking helix step - without energetic input - would be irreversible, able to go only in the backword direction.

Some of the structural detail suggested in the preceding analysis is of course speculative, but the underlying physical principles are certainly correct. In any event, the two chief conformational states required by the model are well established both for Ca^{2+} and Na^+,K^+ pumps. They are generally called the "E_1" and "E_2" states, and do have properties consistent with the model, at least in the case of the SR Ca^{2+} pump. There is high affinity for Ca^{2+} in the E_1 state and low affinity in the E_2 state, a high energy phosphate bond in E_1~P and a very low energy phosphate bond in E_2-P. If ADP is added to E_1~P, then ATP is synthesized - but E_2-P is ADP-insensitive. The optical properties are consistent with the rocking helix model for both Ca^{2+} and Na^+,K^+ pumps: there is a fluorescence change in the reversible transition between E_1 and E_2, but none in circular dichroism. This indicates the absence of a detectable change in α-helix content in the transition. (Nakamoto and Inesi, 1986; Hastings et al, 1986).

An important experimental study done in our own lab-laboratory (Martin et al, 1984) as well as in Aarhus (Andersen et al, 1985) has demonstrated (for the SR Ca^{2+} pump) that the protein is able to carry out all these reactions in a monomeric state - including in particular

the reversible interconversion between Ca_2E_1~P and Ca_2E_2-P, which is the translocation step. There had been several arguments in the literature to suggest that an oligomeric state would be necessary for this part of any ion pump mechanism (e.g. Klingenberg, 1981), but that proved not to be so.

Fig. 1 summarizes our model in a conventional state cycle diagram. In this form the model is not really new: de Meis and coworkers had already proposed it as a kinetic model (without the structural implications) in 1979 (de Meis and Vianna, 1979).

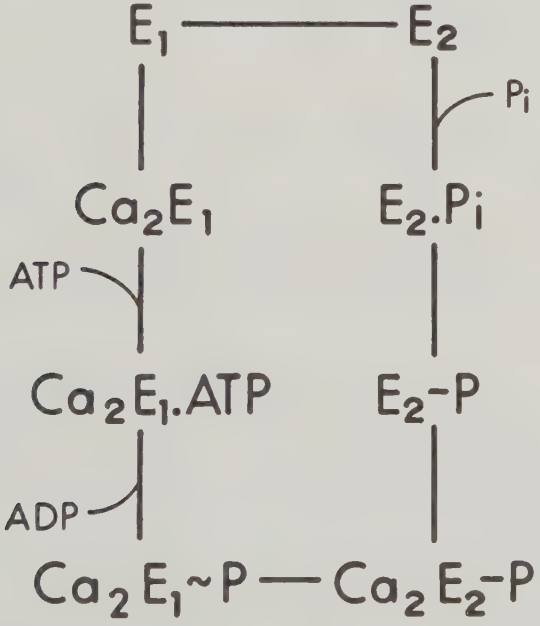

Figure 1. Conventional state diagram cycle for the SR Ca^{2+} pump (de Meis and Vianna, 1979). The focus in the present paper is on the conversion of E_1 to Ca_2E_1 and on the isomerization/translocation step at the bottom of the diagram.

TRANSPORT STOICHIOMETRY

It should be self-evident that the cycle of Fig. 1 can be only a skeletal version of what is happening. The actual number of elementary steps must be much larger than shown. For example, binding Ca^{2+} and ATP is known to occur in random order: ATP can bind in the absence of Ca^{2+} and can even already bind when the protein is still in the E_2 state. Another obvious deficiency is in the binding of Ca^{2+} to E_1. One cannot go straight to Ca_2E_2, but must take up first one Ca^{2+} ion and then a second one in a separate step. That is the part of the cycle on which I want to focus in this section, because that is where the stoichiometry question is localized. Is stoichiometry absolutely fixed, so that the protein with only one bound Ca^{2+} cannot go through the rest of the cycle? If so, how is it done?

TABLE 2. Thermodynamics and Stoichiometry

Ca^{2+}/ATP	Free energy in kcal/mole	
	Available from ATP	Needed for transport*
1	14	6
2	14	12
3	14	18

*The maximal load towards the end of the pumping action, as the resting state Ca^{2+} concentrations are approached.

We begin with Table 2, which shows that there is a thermodynamic limitation on stoichiometry. A 3:1 stoichiometry is impossible - it would lead to reversal of the pump and ATP synthesis under normal physiological conditions. A 1:1 stoichiometry on the other hand would be energetically wasteful. 2:1 is clearly ideally efficient, and that is the stoichiometry that is supported by much of the available experimental data (Hasselbach, 1979).

However, there is no compelling evidence that says that the stoichiometry must be fixed at all. It has in fact been suggested that variable stoichiometry might be

beneficial for physiological function - that a shift to a lower gear at very low Ca^{2+} concentrations, more energy per ion - might speed up the final stages of Ca^{2+} removal from the cytoplasm. The rules of the game actually make that expectation unrealistic. If two alternative translocation pathways are permitted, one with a single bound Ca^{2+}, the other with two, then one cannot avoid also permitting a third cycle (shown in Fig. 2), which is a leak cycle, two Ca^{2+} ions leaving the sarcoplasmic reticulum and only one coming back. And this is what defeats the purpose, prevents one from getting the benefit from shifting gears that might otherwise have been expected (Johnson et al, 1985).

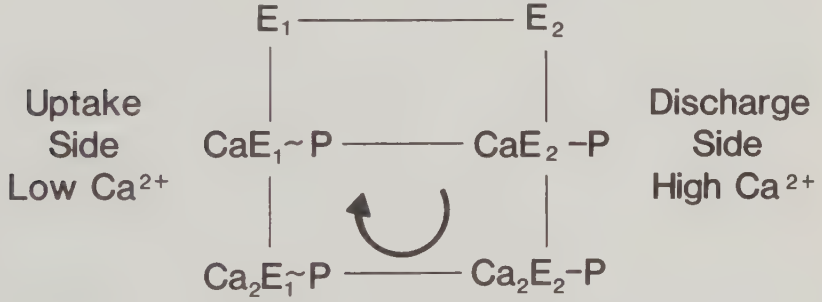

Figure 2. Alternative pathways for isomerization/translocation with different numbers of bound Ca^{2+} ions inevitably generate a leak pathway via the cycle marked by the arrow.

The principle expressed in Fig. 2 of course only tells us that there is no significant advantage to be gained from variable stoichiometry. It does not remove the conceptual difficulty inherent to fixed stoichiometry. In terms of what is going on at the structural level, there is difficulty in imagining how a protein molecule can measure out precisely 2 Ca^{2+} ions or precisely 3 Na^{+} ions each time it goes through its cycle.

EXPERIMENTAL COOPERATIVITY

Before continuing with the elaboration of the model, it is important to look at experimental data on binding of Ca^{2+} to the E_1 state. These data show two quite

different kinds of cooperativity, i.e., steep slopes incompatible with the binding of two Ca^{2+} ions in random order to independent sites in a fixed protein conformation.

One is equilibrium cooperativity. The binding of Ca^{2+} to the pump protein can be studied as an equilibrium process simply by omitting ATP from the reaction medium, so that no cycling is possible. The binding so studied turns out to be formally cooperative - with a Hill coefficient around 1.8 (Inesi et al, 1980; Hill and Inesi, 1982).

The other is kinetic cooperativity, derived from old-fashioned steady state kinetic experiments first done 20 years ago (Hasselbach, 1979). These experiments use leaky vesicles, with the same concentration of Ca^{2+} on both sides of the membrane, i.e. there is no work done in the transport process. Also, the ATP concentration is kept constant. The only thing that is varied is the Ca^{2+} concentration and the only thing that is measured is the rate of ATP hydrolysis. This rate rises steeply at below μM Ca^{2+} as Ca^{2+} uptake is activated, and then the rate falls again at high Ca^{2+} as Ca^{2+} release on the other side of the membrane is blocked. The problem is the slope of the uptake line - it is again a highly cooperative function of $[Ca^{2+}]$ concentration.

Kinetic cooperativity of this kind has a quite different physical origin from equilibrium binding cooperativity. One difference (specific for the present problem) is that ATP is present in the kinetic experiment, whereas it is absent in the equilibrium binding experiment. More generally, the response to increasing Ca^{2+} concentration in a kinetic experiment depends on rate constants and not on equilibrium constants. That introduces a complicating factor - the magnitude of the "on" rate constant for binding of anything to a protein is limited by the rate of diffusion to the binding site. And when the bulk concentration is as low as μM even the maximal diffusion rate is quite slow, which, it turns out, makes it difficult to generate cooperativity in any model calculation for kinetic behavior (Tanford et al, 1985).

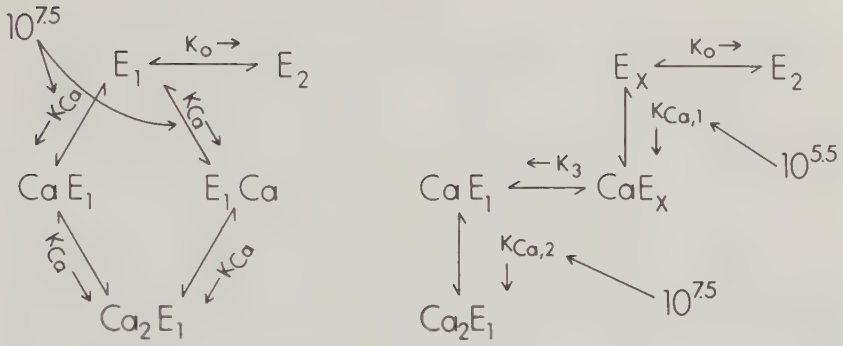

Figure 3. Two schemes for generation of formal cooperativity in the equilibrium binding of Ca^{2+}. The numerical values given for the Ca^{2+} binding constants are approximately the ones needed to fit the binding data of Inesi et al (1980). In scheme 2 the values of $K_{Ca,1}$ and K_3 are interdependent. The number given corresponds to K_3=1.

There are effectively two ways to achieve cooperativity in equilibrium binding, shown in Fig. 3. Scheme 1 is the familiar allosteric mechanism - the two binding sites are actually equally accessible and identical in affinity, but binding of the first ion is more difficult than binding of the second ion because one needs to go through a conformational change (in this case from E_2 to E_1) before any Ca^{2+} can be bound on the uptake side of the cycle.

In scheme 2 we have two different binding sites, the second site having a higher affinity for Ca^{2+} than the first, and this might at first be thought to be a simpler, more direct way to generate cooperativity. In actual fact, however, scheme 2 is more complicated because, if both sites were equally accessible, then the higher affinity site would be filled first (in an equilibrium measurement) as the Ca^{2+} concentration is raised. That would lead to formally negative cooperativity, not the positive cooperativity actually observed. For this scheme to give positive cooperativity, the mechanism must have a compulsory order of binding. In other words, we

must suppose that only the weaker Ca^{2+} binding site is initially available, and that the higher affinity site does not exist until the first site is filled.

Both schemes can fit the equilibrium data equally well, but we intrinsically preferred the simpler possibility given by the allosteric scheme, especially as we already have the requisite two conformational states in the reaction scheme. Unfortunately, however, the kinetic data require scheme 2 - not only to account for cooperativity in steady state activation, but also to account for experimentally measured Ca^{2+} release transients and other fast kinetic data (Inesi, 1985). This made it necessary to try to imagine a structural model that would correspond to scheme 2 - with at least one extra conformational state to take care of the generation of the two binding sites in successive steps.

THE MACLENNAN-GREEN DOMAIN MAP

While this work was in progress, MacLennan and Green and coworkers published their amino acid sequence of the SR Ca^{2+} pump protein, and a functional domain map based on it. (See Fig. 6 below for a modified version.) The map has a bundle of trans-membrane α-helices, presumably containing the translocation pathway for the transported ions. On top of them there is a "stalk" region containing more helices. These helices contain a very large number of negatively charged amino acids. This fact, together with the close proximity to the membrane, led MacLennan and Green to identify the stalk as the calcium binding domain. The ATP binding and phosphorylation domains (to be discussed in the following section) are placed beyond the stalk in the MacLennan-Green diagram, in the cell cytoplasm, a considerable distance from the domain of the bilayer framework of the membrane (MacLennan et al, 1985; Brandl et al, 1986).

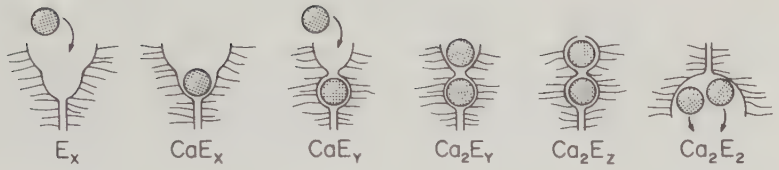

Figure 4. Pictorial representation of the postulated successive stages of the Ca^{2+} binding domain of the protein. The binding cavity is assumed to extend physically into the cytoplasm in the E_1 state and its inner surface is assumed to possess a net negative charge.

The high concentration of negative charges in the designated Ca^{2+} binding region suggested to us a new possibility for how the transported Ca^{2+} ions might be pulled into the binding area of the protein and subsequently handled, a possibility different from that in a postulated transport mechanism given in the MacLennan-Green papers. The basic idea is that electrostatic repulsion between the stalk helices (5 in number) might (when no Ca^{2+} is bound) force the helices apart with the consequent opening up of the binding cavity, like a flower, which then closes only when Ca^{2+} is bound. The overall effect, shown in Fig. 4, is to replace the former single conformation E_1 by three closely related sub-conformations, which I shall call E_x, E_y and E_z. E_x is the open conformation, which will have only a single binding site for Ca^{2+}, with moderately high affinity. When Ca^{2+} is bound, it draws the walls of the cavity together, bringing groups into juxtaposition to create a second site of higher affinity. There is further jaw-closing when the second site is filled, and only after that can the major transition to E_2 take place.

One feature of this model is that (in schematic terms) it is a sequential binding process of the kind I said earlier was needed to account simultaneously for equilibrium and kinetic cooperativity, and indeed it is easy to adjust rate constants for the model to satisfy the experimental data, as shown in Fig. 5.

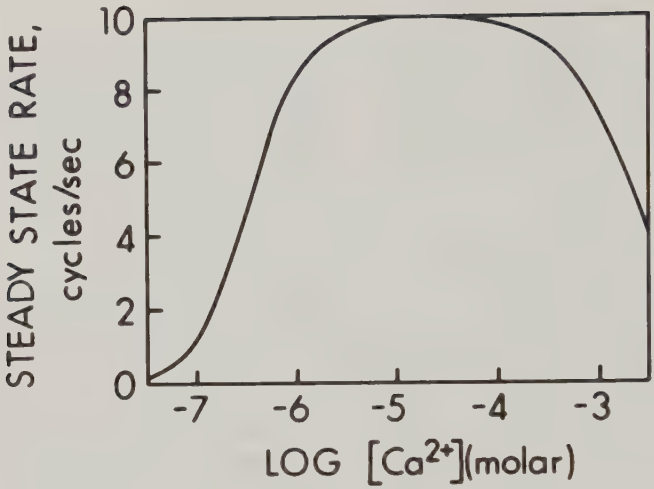

Figure 5. Computer-generated plot of the steady state kinetic data, using parameters identical to those required to reproduce the equilibrium Ca^{2+} binding results of Inesi et al. (1980), and the anomalous ATP activation kinetics (Reynolds et al, 1985a). The upswing line is now as steep as the published experimental curve (Hasselbach, 1979). The inhibition at high $[Ca^{2+}]$ arises from blocking Ca^{2+} release from Ca_2E_2-P.

The model of Fig. 4, in addition to satisfying kinetic requirements, also turns out to answer the question of stoichiometry. I said before that it was difficult for us to imagine a plausible way to devise a constant stoichiometry machine, but the model clearly does that. There is now no way to go to the $Ca_2E\sim P$ state and beyond without having precisely two Ca^{2+} ions bound. Also note that the two bound Ca^{2+} ions in state $Ca_2E\sim P$ are now "occluded", not accessible from either side of the membrane, which is in complete accord with experimental findings (Dupont, 1980; Takisawa and Makinose, 1983).

STRUCTURAL COUPLING BETWEEN ATP PROCESSING AND ION TRANSLOCATION.

I said at the beginning of this paper that we were not planning to attempt an approach to the energetic coupling

problem until after we felt that we had a satisfactory postulate for the ion translocation mechanism by itself, independent of the energy source to which it might be coupled. As things turned out, no new intellectual effort was needed at all to postulate a plausible coupling mechanism. The translocation mechanism in itself suggested how the ATP processing and ion translocation domains might be structurally linked, a delightful example of serendipity.[1]

Previous work had shown that the high affinity ATP binding region of the protein is in a different part of the sequence from the aspartyl residue (number 351 in the sequence) to which the terminal phosphate group of the ATP is subsequently covalently bound (Mitchinson et al, 1982). The corresponding domains are shown in Fig. 6. Green and MacLennan and their coworkers recognized the need for a hinge somewhere in the structure to move the ATP binding site into contact with the aspartic acid residue to which the γ-phosphate group of ATP is to be transferred. In their model they speculatively put the hinge in the position shown in the Figure.

But in our model we already have a hinge somewhere else - at the bottom of the binding cavity. What if this hinge serves dual purposes - both to close the binding cavity and to bring these sites together? That clearly provides an obligatory link between the ATP processing sites and Ca^{2+} binding. Simple ATP binding is still independent of whether or not Ca^{2+} is present, but phosphorylation now becomes absolutely dependent on Ca^{2+} - cannot occur unless 2 Ca^{2+} ions are bound first. And that is consistent with the experimental facts, and is in fact a fine detail of the experimental facts that had previously seemed rather mysterious.

[1] The dictionary definition of this useful English word is the lucky ability to find something more valuable and agreeable than what you were actually seeking. The word has no familiar roots in other European languages because it was coined in 1754 by the English writer, Horace Walpole, on the basis of the plot of a *Persian* fairy tale, "The three princes of Serendip", who were fortunate possessors of this gift. *Serendip* itself is an ancient *Arab* word for *Ceylon*, which is now (even in English) called *Sri Lanka*.

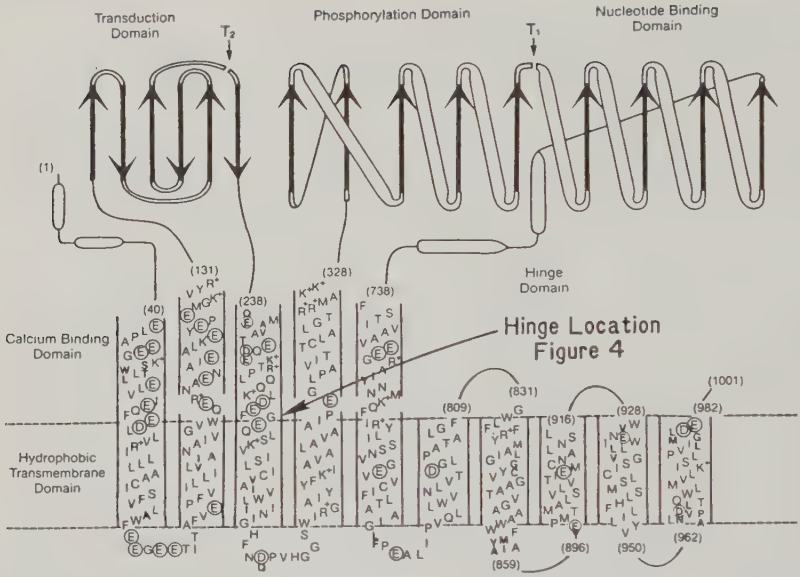

Figure 6. Structural diagram of the SR Ca^{2+} pump molecule based on the amino acid sequence, hydrophobicity plots, and predicted domain arrangement, from Brandl et al. (1986). The words "hinge domain" identify the hinge location suggested in the original paper. The arrow points to the hinge location suggested here.

Our dual purpose hinge is of course just as speculative as MacLennan and Green's hinge. But the serendipity - everything falling into place quite unintentionally - lends credibility to the theoretical model. Usually, when a simple theoretical explanation for some phenomenon has been worked out, it soon turns out to be incompatible with some related phenomenon and awkward (less simple) adjustments tend to be required to make it compatible. In this way initial enthusiasm for many theories often becomes quickly tarnished. The situation here is the opposite, the theory gives us more than we initially asked from it.

DISCUSSION: RELATION TO Na^+,K^+ PUMPS

The similarities between the SR Ca^{2+} pump and Na^+,K^+ pumps from many different membranes are well known. The E_1 and E_2 conformations, for example, are alike for the two

kinds of protein, and so are the corresponding high and low energy phosphoenzyme states. The entire skeletal cycle for the Ca^{2+} pump shown in Fig. 1 is closely similar to the basic Post-Albers scheme for the Na^+,K^+ pump. These similarities have been reinforced by recent structural work. The first primary sequences for catalytic α-chains of Na^+, K^+ ATPases were determined almost simultaneously with the determination of the primary structure of the SR Ca^{2+} pump, and, as expected, similar patterns of predicted domain arrangements have emerged (Kawakami et al, 1985; Shull et al, 1985).

There are also, however, differences between the Ca^{2+} and Na^+,K^+ proteins. The most striking one is that the Na^+,K^+ pump protein possesses a second polypeptide chain, the glycosylated β-chain. Its functional role is not yet established, but its presence alone suffices to suggest the exercise of caution when mechanisms of the two pumps are compared.

There are also more tangible differences. It might even be appropriate to call them more interesting differences from the point of view of the mechanism put forward in this paper, because they generate new questions to be asked, which are valid even if the β-chain turns out to have no direct role in the catalytic mechanism at all and even if the transport and free energy transduction mechanisms for the Ca^{2+} and Na^+,K^+ systems turn out to be identical. In fact, the newly generated questions are especially timely if the latter circumstance is correct, because they would then provide tests for a communal, perhaps quite general "ATP-driven ion pump" mechanism that one cannot apply to the SR Ca^{2+} system alone. Here are two examples:

(1) The alternating access concept of course necessitates two conformational flips per cycle, one from inside to outside (which in our model and in the Post-Albers scheme for Na^+,K^+ transport involves the phosphorylated forms of E_1 and E_2), and then the outside to inside flip (unphosphorylated E_2 to E_1). What do we imagine is happening to the ion binding site in the latter step? Our analysis of this question for the SR Ca^{2+} pump has been most casual; nothing approaching the detail given in Fig. 4 has been attempted. There is a good reason for this: in the case of the SR Ca^{2+} pump there appear to be no necessary ions bound to the protein in the E_2 to E_1 return, and there is consequently no experimental handle for direct study of

the changes in the ion binding site that accompany the E_2 to E_1 transition. The situation is quite different for the Na^+,K^+ pump. There is obligatory transport of K^+ on the return flip and, in the normal Post-Albers cycle, state E_2 even contains occluded K^+. Does that imply sub-states of E_2 like the ones for E_1 in Fig. 4?

(2) Most Na^+,K^+ pumps are located in cytoplasmic membranes that support an electrical membrane potential. This opens up an entire new dimension for experimental studies. All thermodynamic and kinetic parameters can now be measured as a function of an applied membrane potential. A theoretical basis for interpreting such measurements (in relation to a structure-based mechanism) has been presented (Reynolds et al, 1985b). The point to be made here is that this provides a new means for testing a mechanism. New data and new theoretical principles are brought to bear, but there is no room for new chemical steps in the mechanism.

As noted earlier in relation to equation 1, the existence of a membrane potential also raises fundamental thermodynamic questions. Consider a binding site for an ion. Even suppose that its three-dimensional structure and relation to the protein surface is definitely established. What is the relation between the pure chemical potential of an ion bound to the site and the corresponding electrochemical potential as a function of voltage? The answer clearly affects how we must think about the relationship between the work of transport and structural changes in the binding site, as was done in Table 1, but there has been little theoretical work on this problem, and the question is at present unanswerable.

Acknowledgments

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OVERVIEW:
STOICHIOMETRY AND VOLTAGE DEPENDENCE OF THE Na/K PUMP

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In this chapter we briefly review recent evidence bearing on the stoichiometry of Na/K transport by the sodium pump and the possibility of its variability, on the voltage dependence of the rate of pumping, a necessary consequence of unequal stoichiometry, and on the identity of the reaction step(s) conferring that voltage sensitivity.

STOICHIOMETRY OF THE Na/K PUMP

Since the early work of Post and Jolly (1957) on the Na/K pump in red blood cells, most other investigators have either confirmed their finding of a 3Na:2K stoichiometry or, at least, obtained results "compatible" with that stoichiometry. As Glynn (1984) put it, "the statement that, under physiological conditions, the ratio of sodium ions pumped out to potassium ions pumped in is 3:2 is sometimes made in a manner more appropriate to the recitation of a creed than to the recounting of an experimental finding". The major problem is that it is still difficult to make simultaneous accurate measurements of uptake and efflux in suspensions of cells such as erythrocytes. Different technical problems arise with single cells, the most serious being the measurement of influxes. A problem of interpretation, particularly grave in excitable cells, comes from the high sensitivity of passive K flux to changes in both voltage and K concentration; yet papers regularly appear in which pumped K influx is estimated simply as ouabain-sensitive K influx,

without further controls. From plain flux studies then, no major news can be added to that reported by Post and Jolly (1957) and by Garrahan and Glynn (1967). The techniques remain difficult, and no obvious improvements are in sight.

Ion activation curves in red blood cells and skeletal muscle fibers have long been known to be sigmoid. Although not an obligatory consequence of multiple stoichiometry, it is rather gratifying to find a Hill coefficient larger than one (or two) and experimenters rarely pass up an opportunity to publish sigmoid curves. Recently, even such bastions of linearity as the squid giant axon (Kracke and De Weer, 1982) and heart muscle (Sejersted et al, 1988) have yielded clearly sigmoid and saturating curves for pump activity as a function of $[Na]_i$. How could these sigmoid relationships have escaped attention for so long? Technical difficulties aside, two reasons stand out. The major reason for believing that Na efflux from squid axon varied linearly with $[Na]_i$ came from an experiment of Hodgkin and Keynes (1956) in which they injected cold Na into an axon extruding labeled Na and saw no change in efflux rate constant, implying linearity. Generally, however, two concentrations on a sigmoid activation curve will yield identical rate constants. By coincidence, Hodgkin and Keynes must have injected just enough unlabeled sodium to raise the concentration to the other point with the same rate constant. A second reason is that sigmoid curves can look uncannily linear over relatively wide concentration ranges. The news from Na activation curves, then, is that they all seem to turn out sigmoid, consistent with multiple stoichiometry, and that apparent linearity of Na efflux with $[Na]_i$ under certain conditions has a reasonable explanation.

Instead of measuring both pump fluxes, another way to determine pump stoichiometry is to measure one of the fluxes as well as the pump current, using a "pump clamp" technique, simple in principle if not in execution. When a cell is voltage-clamped at its resting potential, the clamp circuitry need pass no current, but sudden arrest of the electrogenic Na/K pump (say with ouabain) requires the circuitry to pass a current equal to that generated by the pump before its arrest. The clamp must be both sensitive and stable since these current changes are relatively small and slow: 3-4 orders of magnitude lower in intensity and speed than usual channel currents in excitable cells, and even an order of magnitude smaller than typical channel

gating currents. If net pump current is measured together with pumped Na efflux (easier to measure than K influx), the pump's stoichiometry can be simply computed. There are three technical challenges: (1) there should be no electroneutral exchange contaminating the flux; (2) the membrane surface areas for current and flux measurements must be identical (no problem if the entire cell surface is involved); and (3) ouabain-sensitive current must represent pump current and nothing else. This last requirement is far from trivial, especially in excitable cells with unstirred extracellular spaces where K may accumulate, altering membrane K conductance, when the pump is stopped. The first to use this approach were Cooke et al (1974) in Paris, who found a Na/K ratio of 2:1 in snail neurons. Rakowski and De Weer (1982) in Woods Hole made similar measurements on squid axons and also found a 2:1 ratio. Figure 1 shows the result of such an experiment where, although membrane areas for flux and current measurements were matched to within 5%, the flux: current ratio was 2.2:1 rather than the 3:1 expected for 3:2 Na/K stoichiometry. We now believe that these current shifts, and those of Cooke et al (1974), overestimated pump current because of contaminating changes in K current, since in more recent experiments (e.g. De Weer et al, 1987) in which K conductance was practically eliminated with intra-

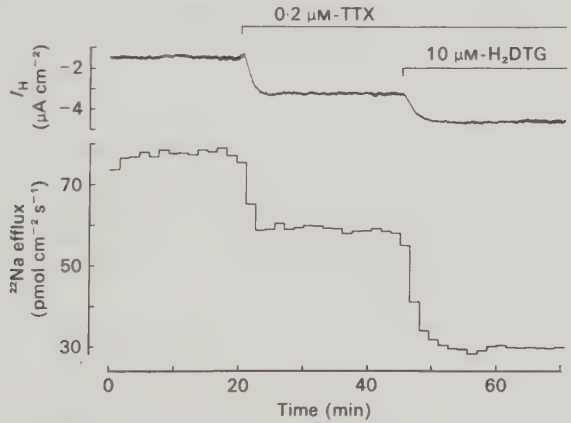

Figure 1. Simultaneous measurement, in a squid giant axon, of changes in holding current (I_H) and in ^{22}Na efflux, at 0 mV, caused by tetrodotoxin (TTX) and dihydrodigitoxigenin (H_2DTG). The axon was internally dialyzed with K-free solution containing (mM) 50 Na, 5 ATP, and 0.25 veratridine, and superfused with 10 mM-K, Na-free seawater. From De Weer et al (1986), with permission.

cellular TEA (200 mM) and phenylpropyl-triethylammonium (20 mM) we find the pump current to be very nearly 1/3 of the Na efflux. Results from Xenopus oocytes obtained by Eisner et al (1987) in London also show a pump current about 1/3 the size of the strophanthidin-sensitive Na efflux. The conclusion to be drawn from these pump-clamp experiments then is that, if proper precautions are taken, cells other than red cells can be demonstrated to have 3Na:2K pump stoichiometry.

Is the pump's stoichiometry variable? Probably not. First, some of the data that suggested variable stoichiometry, e.g. a linear dependence of pump rate on $[Na]_i$ but saturating dependence on $[K]_o$, are no longer tenable. Secondly, there is reasonable evidence that pump stoichiometry is independent of $[Na]_i$ (Thomas, 1969; Gadsby and Cranefield, 1979; Eisner and Lederer, 1980; Glitsch and Pusch, 1980; Eisner et al, 1981a; Glitsch et al, 1982) or of $[K]_o$ (or $[Rb]_o$ or $[Cs]_o$) (Abercrombie and De Weer, 1978; Gadsby, 1980; Eisner and Lederer, 1980; Eisner et al, 1981b) or of internal [ATP] (Goldschleger et al, 1987). Thirdly, in squid giant axons, the stoichiometry seems independent of $[Na]_o$ and of membrane potential between -60 and 0 mV (De Weer et al, 1987). And lastly, even in the most extreme circumstance, running backwards, we find that the pump is still electrogenic (De Weer and Rakowski, 1984; Rakowski et al, 1988; Bahinski et al, 1988) transporting Na inward and K outward in what appears to be a 3:2 ratio (De Weer and Rakowski, 1984). Thus, until new and compelling evidence arises, the pump stoichiometry seems to be fixed at 3:2, independent of potential, of external K or internal or external Na concentrations, and of whether the pump runs forwards or backwards. Admittedly, this brief analysis has ignored the intriguing finding of Dissing and Hoffman (1983) that under extreme conditions anions may accompany Na ions across the pump machinery.

VOLTAGE DEPENDENCE OF THE Na/K PUMP

The unequal stoichiometry, and consequent extrusion of net charge by the pump, requires the pump rate to be voltage dependent (for review, see De Weer et al, 1988). An electrogenic pump can be represented by a chemical battery with an electromotive force, $E_{Na/K}$, equal to the experimental reversal potential, i.e. the membrane potential at which the pump produces no current. $E_{Na/K}$ is a function of the free

energy of hydrolysis of ATP, of the Na and K gradients, and of the Na/K stoichiometry:

$$E_{Na/K} = (E_{ATP} + mE_{Na} - nE_K)/(m - n) \qquad \text{(eqn. 1)}$$

where E_{ATP} reflects the free energy of ATP hydrolysis, E_{Na} and E_K are the respective equilibrium potentials, and m and n are the pump's stoichiometric coefficients. With the Na and K gradients of a typical animal cell $E_{Na/K}$ will be in the vicinity of -200 mV so that, obviously, pump current must vary with voltage somewhere between the reversal potential and the resting potential.

Can the form of this voltage dependence be predicted? Unfortunately not yet, because not enough is known about the possible voltage sensitivity of the many steps in the pump reaction cycle. Even the most reductionist models, those with only two states and two steps, can produce a wide variety of current-voltage curves depending on the choice of parameters. Theory nevertheless provides a very useful diagnostic constraint: for an unbranched cycle without gating, the voltage dependence will be monotonic if there is a single voltage-sensitive step, but must be biphasic (i.e. include a region of negative slope) if a second voltage-sensitive step involves an opposing charge movement (Gradmann et al, 1982). Fortunately, experimental data are now helping us identify the voltage-dependent step(s) in the pump cycle, with evidence from four sources: squid axon, Xenopus oocytes, heart cells, and isolated (Na,K)ATPase.

We begin with squid axon because we work on it, and we have a retraction to make. Figure 2 shows a pump current-voltage relationship obtained by subtracting currents recorded before and after application of dihydrodigitoxigenin to a squid axon whose ion conductances had been greatly reduced by various channel blockers (De Weer et al, 1987). Pump current declines with hyperpolarization, as expected since the pump must work harder, but the reversal potential is well beyond experimental range. The drop in current is not due to a change in stoichiometry because, as already mentioned, the pumped Na efflux is equally voltage sensitive (De Weer et al, 1987). Removal of external Na increases pump current (and pumped Na efflux) at large negative potentials, leaving the pump rate only weakly voltage-dependent (De Weer et al, 1987). While equation 1 predicts a large leftward shift of the reversal potential (theoretically, to minus infinity), there is no a priori kinetic reason for the nearly voltage-independent behavior

of the pump in Na-free medium. This behavior must mean that whatever step limits cycling rate in the presence of external Na, no longer does so in its absence.

As for the retraction: De Weer and Rakowski (1984) had reported a voltage dependence of the backward-running pump indicating a region of negative slope conductance, consistent with the presence of two opposing voltage-sensitive

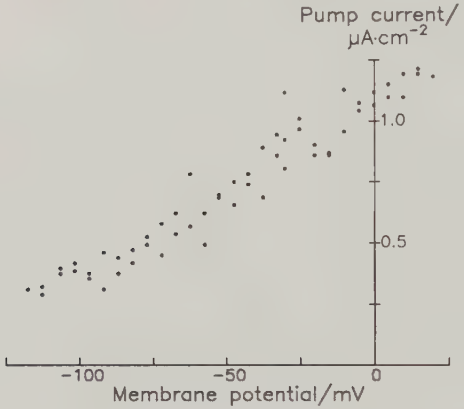

Figure 2. Voltage dependence of Na/K pump current in a squid giant axon bathed in 10 mM-K, 400 mM-Na seawater and internally dialyzed with 50 mM Na and 5 mM ATP.

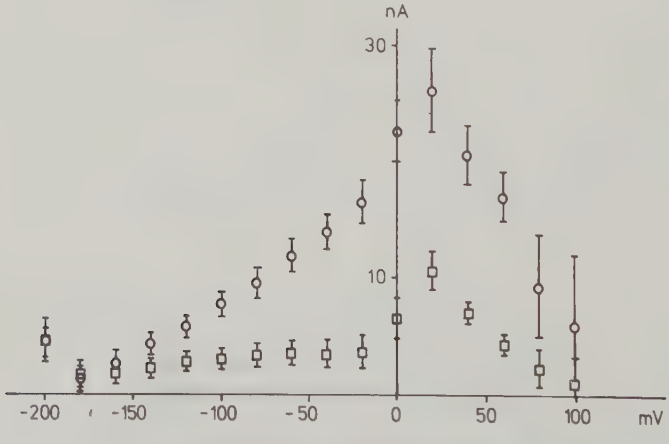

Figure 3. Voltage dependence of Na/K pump current in Xenopus oocytes with elevated (circles) or normal (squares) $[\overline{Na}]_i$. From Lafaire and Schwarz (1986), with permission.

steps. The result turns out to be attributable to incompletely controlled "end-effects", and we have recently shown the current-voltage relationship of the backward-running pump to be a monotonic curve with no negative slope, but with a plateau at the most negative potentials (Rakowski et al, 1988).

Turning now to oocytes, Figure 3 shows results from the work of Lafaire and Schwarz (1986) in Frankfurt. The dihydroouabain-sensitive current-voltage curve shows a clear peak and then a negative slope region at positive potentials, that is, the pump seems to slow as the going gets easier. As stated earlier, this could be caused by two voltage-sensitive steps in the reaction cycle, the second moving charge in the opposite sense to the first, and we found this idea attractive because of our earlier belief that the backward-running pump curve also included a negative slope region. In similar experiments with oocytes, Eisner et al (1987) found no negative slope, but their data stop short of positive potentials. Oocytes seem to suffer from one of the technical problems mentioned above, a K current very sensitive to $[K]_o$ (Lafaire and Schwarz, 1986), so that a small accumulation of K in the extracellular space can cause a measurable change in non-pump current. Rakowski (1987) found that, in Xenopus oocytes, cardiotonic steroid-sensitive current-voltage curves that do not show hysteresis are monotonic between -180 and +50 mV and saturate at positive potentials. We now prefer to believe, therefore, that neither the forward- nor the backward-running pump current-voltage relationship includes a negative slope region, and so there is no reason to postulate more than one voltage-sensitive step.

Similar monotonic voltage dependence has been seen in other preparations. For example, the turnover rate of (Na,K)ATPase reconstituted into lipid vesicles is monotonically voltage sensitive (Goldshlegger et al, 1987). Additional data on the voltage dependence of Na/K pump current in vivo are emerging from experiments on isolated heart cells (Gadsby et al, 1985; Nakao and Gadsby, 1986; Gadsby and Nakao, 1987; Bahinski et al, 1988). Here again (Fig. 4) the pump current is diminished by hyperpolarization (but less so at low external [Na]) and levels off at positive potentials. When the pump is made to run backwards (Fig. 5) pump current is again monotonically voltage dependent, leveling off at negative potentials.

The conclusions to be drawn, then, are: (1) the Na/K pump is voltage sensitive, monotonically speeding up and saturating at large positive potentials for forward pumping and monotonically speeding up and saturating at large negative potentials for backward pumping; (2) a single voltage-sensitive step is required to account for these findings.

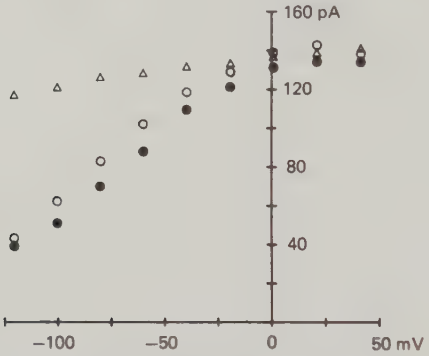

Figure 4. Current-voltage relationships of forward-running Na/K pump in a single internally-dialyzed cell from guinea-pig ventricle, obtained as the difference between steady-state membrane currents recorded in the absence or presence of 2 mM strophanthidin. $[Na]_i$ was 50 mM and $[K]_o$ 5.4 mM. External [Na] was either 150 mM (circles) or 1.5 mM (triangles). From Gadsby and Nakao (1987), with permission.

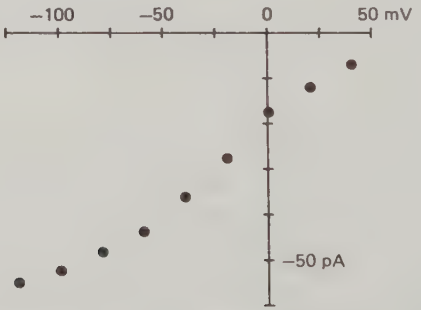

Figure 5. Current-voltage relationship of backward-running Na/K pump in a single cell from guinea-pig ventricle, obtained as in Fig. 4. The cell was internally dialyzed with Na-free solution containing 5 mM each of ATP, ADP, and P_i, and bathed in K-free, 150 mM-Na fluid. From Bahinski et al (1988), with permission.

NATURE OF THE VOLTAGE-DEPENDENT STEP(S)

Present evidence from work on reconstituted pumps in vesicles, on pumps attached to plane lipid bilayers, and on pumps in isolated cardiac myocytes, strongly suggests that the Na translocation step is the major voltage-sensitive event in the pump reaction cycle. Karlish and coworkers in Israel used reconstituted vesicles with imposed ionophore-induced membrane potentials. At micromolar ATP levels, when K deocclusion is believed rate-limiting, the Na/K transport rate was insensitive to voltage, whereas it was voltage sensitive at high ATP levels. Hence K deocclusion probably is not voltage sensitive, but the Na translocation step possibly is (Goldshlegger et al, 1987). In support of the former conclusion, they found K/K exchange (monitored as Rb/Rb exchange) to be unaffected by voltage, as were the rates of transitions between the K-loaded forms of E_1 and E_2 measured by fluorescence (Rephaeli et al, 1986a); and, in support of the latter conclusion, fluorescence measurements indicated that E_1 to E_2 transitions of Na-loaded enzyme in the presence of acetylphosphate were accelerated by a positive potential (Rephaeli et al, 1986b).

Bamberg and coworkers in Frankfurt (Fendler et al, 1985, 1987; Nagel et al, 1987) attached (Na,K)ATPase-containing membrane fragments to plane lipid bilayers, and photolyzed caged ATP with a flash of light to start the pump reaction. Under short-circuit conditions (i.e. voltage-clamped at 0 mV) they saw a transient current which required Na but not K, and was inhibited by ouabain. In similar experiments, Läuger and coworkers in Konstanz (Borlinghaus et al, 1987; Apell et al, 1987) recorded the same rapidly rising and slowly decaying transient pump current. Both groups concluded that the reaction involved an electrically silent step (or steps) followed by an electrogenic step, but it is not yet agreed whether the faster step precedes (Apell et al, 1987) or follows (Fendler et al, 1987) the slower one. The Konstanz group went on to treat some (Na,K)ATPase with chymotrypsin which leaves the enzyme capable of occluding Na but incapable of the transition to E_2 from which Na is released: proteolysis abolished the transient currents, despite the persistence (presumably) of Na occlusion. Hence the charge-carrying step must occur after Na occlusion, and so is likely to be either the E_1-E_2 conformational change or the Na-releasing step.

If a step in the pump cycle involves translocation of charge, then a sudden jump of membrane potential can cause membrane charge movements, reflecting a redistribution of pump species (Hansen et al, 1983) and measurable as a transient pump current (sometimes called displacement current or gating current). This is what Nakao and Gadsby

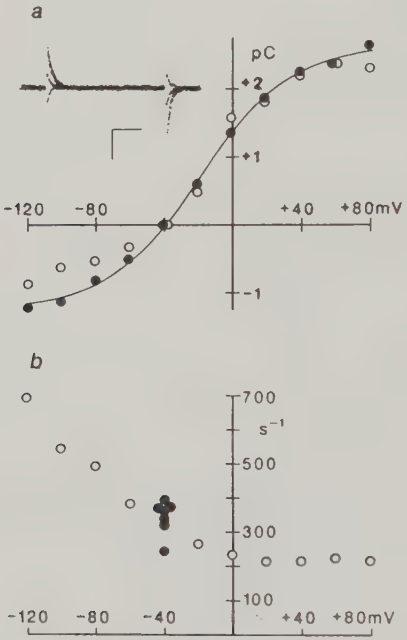

Figure 6. Voltage dependence of charge movement (a) and equilibration rate constant (b) during strophanthidin-sensitive current transients (obtained by computer subtraction and shown superimposed in inset a) elicited by 100-ms voltage pulses from -40 mV, in a single cell from guinea-pig ventricle, internally dialyzed with fluid containing (mM) 50 Na and 10 ATP, and bathed in K-free, 150 mM-Na solution. In panel (a) are plotted the time integrals Q of the "on" transient currents; the equation of the curve describing the relationship between Q and membrane potential (V) during the pulse is $Q = Q_{min} + Q_{tot}/[1 + \exp(V'-V)/k]$, where k is 26.5 mV. Panel (b) shows the rate constants of exponential fits to the equilibration transients: open circles, "on" transients; closed circles, "off" transients upon return to -40 mV holding potential. Further description in text. From Nakao and Gadsby (1986), with permission.

(1986) recorded in cardiac myocytes whose pumps, due to lack of K, were restricted to Na/Na exchange (Fig. 6a, inset). From a holding potential of -40 mV, pulses of increasing size in the depolarizing or hyperpolarizing direction caused outward or inward movements of increasing amounts of charge which eventually saturated in both directions. The charge-voltage curve (Fig. 6a) is well approximated by a Boltzmann distribution with three adjustable parameters: (1) the midpoint V′, here -20 mV, at which half of the mobile charges are on either side; (2) the vertical distance Q_{tot} between the saturation levels, reflecting the total number of mobile charges, here about 1000 elementary charges per square micron; and (3) the steepness of the curve, reflecting the size of the charge on the mobile species, here one electronic charge (assuming movement through the entire membrane field). That also means there must be 1000 pumps per square micron, in excellent agreement with ouabain binding measurements (Lobaugh and Lieberman, 1987). Figure 6b shows the voltage dependence of the equilibration rate constants (i.e. for exponential decay of the net charge movement) which are seen to be high for negative potentials and low for positive potentials. Since these equilibration rate constants are the sums of the forward and backward transition rate constants, it is obvious that the backward rate (increased by hyperpolarization) is much more affected by voltage than the forward rate or, in the terminology of Eyring barrier models (Läuger and Stark, 1970), that the charge translocation occurs over a very asymmetric barrier.

CONCLUSION

The evidence reviewed here shows that: the electrogenic Na/K pump is voltage sensitive; the I-V curves (excepting some data on oocytes) are monotonic, consistent with a single voltage-sensitive step; this step is from overwhelming evidence the Na translocation step; it probably occurs after occlusion but before release of Na; it involves one electronic charge per event (consistent with K translocation being electroneutral); and voltage affects the backward more than the forward rate constant.

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OVERVIEW:
THE COUPLING OF ENZYMATIC STEPS TO THE TRANSLOCATION OF SODIUM AND POTASSIUM

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INTRODUCTION

The title of this talk was suggested by the Organizing Committee. It seems to me an ambitious one, suggesting perhaps, that we know how the pump works. Of course, we do not, and all I can do is to give a sketchy outline of the development of current views, and to draw attention to certain awkward problems.

THE 1978 SCHEME

It is convenient to start with an overall scheme that we proposed nine years ago (Karlish *et al.* 1978) - see Figure 1. That scheme is essentially the extended Albers-Post scheme - with two forms of phosphoenzyme E_1P and E_2P, and the occluded-K form $E_2(K)$ - but grafted onto it are extra hypotheses about the accessibility of the ion-binding sites from the two surfaces of the membrane. (In talking about ion-binding sites here, we are concerned only with the sites that bind the transported ions - the cation-loading sites. There are, of course, other sites at which sodium and potassium ions may combine and have effects.) The first extra hypothesis is that it is only the unphosphorylated enzyme in the E_1 form that has cation-loading sites accessible from the interior of the cell; those sites are supposed to prefer sodium to potassium. The second hypothesis is that it is only phosphorylated enzyme in the E_2P form that has cation-loading sites accessible from

the exterior; those sites are supposed to prefer potassium to sodium.

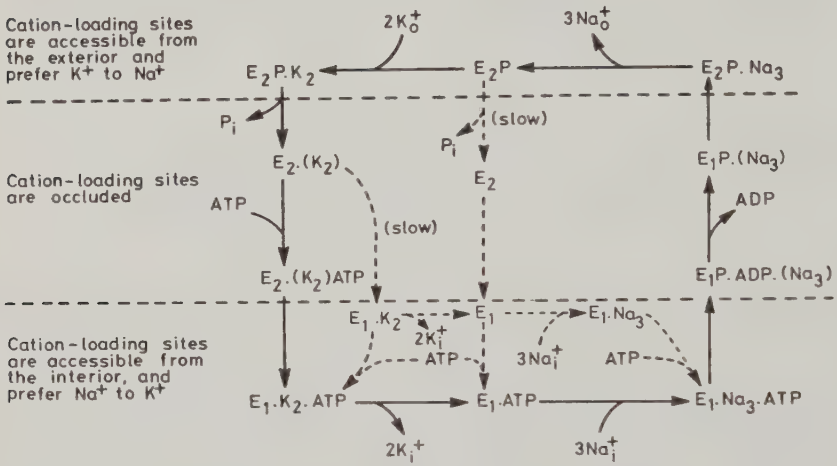

Figure 1. A scheme, modified from Karlish *et al.* (1978), showing the proposed relations beteen ion movements, transfers of phospho groups, and conformational changes. The solid arrows show the normal cycle. Occluded ions are shown in brackets.

At the time this scheme was put forward it was based on evidence of five different kinds - for a fuller account see review by Glynn (1985). First, there were studies of phosphorylation and dephosphorylation - both the studies that had led to the original Albers-Post scheme (Fahn *et al.* 1966a,b; Siegel and Albers, 1967; Post *et al.* 1969) and the subsequent studies showing that the properties of newly dephosphorylated enzyme depended on the ion catalysing the dephosphorylation (Post *et al.* 1972). Secondly, the scheme was based on a large number of flux studies showing that under different conditions the pump can operate in a variety of different modes - see review by Glynn (1985). These studies had defined the conditions necessary for operation in each mode and the biochemical changes which accompany each mode. Thirdly, studies of nucleotide binding (Hegyvary and Post, 1971; Nørby and Jensen, 1971) had shown that the

dephosphoenzyme can exist in two forms with very different affinities. Fourthly, studies of the patterns of proteolytic digestion (Jørgensen, 1975,1977) had shown that both phospho and dephospho forms can exist in more than one conformation. Lastly, the scheme was based on stopped-flow fluorimetry experiments using the fluorescent formycin analogues of ATP and ADP (Karlish *et al.* 1978). These experiments provided clear evidence that the dephospho form stable in a potassium medium is converted only very slowly to the dephospho form stable in a sodium medium, and that that conversion is greatly accelerated by nucleotides. The nucleotides act with a low affinity and their action does not involve phosphorylation.

During the last nine years there have, of course, been many developments. Some of these greatly strengthen the scheme; some require it to be modified, and some appear to throw doubt on its validity. In what follows, I shall discuss some of these developments briefly, starting with those most favourable to the scheme and working round to the most awkward.

FURTHER EVIDENCE FOR THE SCHEME

Direct Evidence for Occluded-Ion Forms

The most dramatic experiments supporting the scheme are those providing direct evidence for the existence of the hypothetical occluded-ion forms. If the conversion of E_1P to E_2P is blocked - by pre-treating the enzyme either with n-ethylmaleimide or with *alpha* chymotrypsin - then it is possible to demonstrate that phosphorylation by ATP leads to the occlusion of three sodium ions in the E_1P form of the phosphoenzyme (Glynn *et al.* 1984). The phosphorylated enzyme can carry the sodium ions through an ion-exchange column. A similar result has recently been observed in experiments in which *beta,gamma*-bidentate CrATP was used as an analogue for MgATP (Vilsen *et al.* this volume). Schoner *et al.* (1983) have shown that the phosphoenzyme generated by CrATP behaves as if it is trapped in the E_1P conformation.

In the potassium half of the cycle, it is possible to demonstrate occlusion even without modifying the enzyme (Beaugé and Glynn, 1979a; Glynn and Richards, 1982). (^{86}Rb is generally used as a tracer for potassium in these

experiments, since it is obtainable with a much higher specific activity than ^{42}K, and much more cheaply than ^{43}K.) In the absence of ATP, or when the ATP level is very low, the conversion of E_2 to E_1 is so slow that it is possible to pass E_2 containing occluded rubidium ions through a resin column quickly enough for it to emerge before more than a small fraction of the enzyme has changed conformation. Furthermore, it is possible to demonstrate that the occluded potassium form can, as the scheme suggests, be approached by two routes - Figure 2. $E_2(Rb)$ can be formed either by adding rubidium ions directly to the enzyme in a sodium-free medium, or by allowing rubidium ions to catalyse the hydrolysis of E_2P made by exposing the enzyme to ATP in a sodium medium - in other words by following what, if the scheme is correct, is the physiological route.

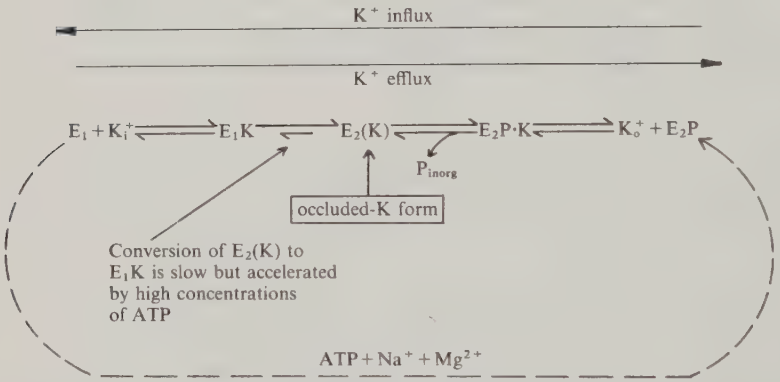

Figure 2. Scheme (reproduced from Glynn *et al.* 1985b) showing that the occluded potassium form of the dephospho-enzyme can be generated *either* by potassium-catalysed hydrolysis of the phosphoenzyme - the physiological route - *or*, directly, by the addition of potassium to the dephosphoenzyme in appropriate conditions. (The stoichiometry of ion-binding and occlusion is not shown.)

An important feature of the scheme is that in the physiological route the rubidium ions act with a high affinity and from the outside surface, whereas in the direct route they act with a low affinity and from the inside

surface. Although a direct test is not possible, there is strong indirect evidence for these differences in affinity and sidedness. The work of Blostein and Chu (1977) shows that potassium ions that catalyse the hydrolysis of E_2P act at high-affinity extracellular sites. It is presumably these ions that become occluded when occlusion is by the physiological route. When occlusion is by the direct route, the ions bind, initially, to sites on E_1. Work with formycin nucleotides (Karlish *et al.* 1978) and studies of intrinsic fluorescence (Beaugé and Glynn, 1980; Jørgensen and Karlish, 1980) strongly suggest that these sites have a low affinity for potassium ions. Evidence that they are intracellular comes from experiments of Karlish and Pick (1981) on the effects of potassium and sodium on tryptic digestion patterns of unphosphorylated Na,K-ATPase incorporated into the membranes of artificial lipid vesicles. Karlish and Pick found that only potassium ions that had access to the intracellular surface could convert the unphosphorylated enzyme to the form giving the tryptic digestion pattern characteristic of potassium media.

We shall come back to the properties of occluded ion forms at the end of this talk, but it is convenient, first, to consider other evidence bearing on the scheme.

Evidence from Flux Studies

Strong support for the scheme comes from a series of flux studies by Sachs (1980, 1981, 1986a,b). These include studies of the effects of vanadate and oligomycin, and a detailed study of potassium:potassium exchange which led Sachs first to predict the existence of, and then to demonstrate, an uncoupled potassium efflux. Further support is provided by the single turnover experiments of Forbush (1984,1985). He prepared right-side-out membrane vesicles from dog kidney, preloaded them with caged ATP and labelled sodium, and then exposed them to flashes of light. He found that a flash or series of flashes that released fewer ATP molecules than the number of catalytic sites caused a burst of ^{22}Na extrusion, and that the size of the burst was unaffected by the presence of potassium. This is the kind of behaviour predicted by models of the pump in which sodium is released early in the cycle. Karlish and Kaplan (1985) have also shown a burst of ^{22}Na transport associated with a

single turnover of Na,K-ATPase incorporated into the membranes of artificial lipid vesicles.

Evidence from Inhibitor Studies

Another line of work supporting the scheme is that by Fortes and Lee (1984), who used the rate of binding of anthroylouabain, and the effect of oligomycin on that rate, to estimate, indirectly, the levels of E_1P and E_2P under different conditions. More specifically, they looked at the rates with and without potassium, and at different levels of ATP. They found that the rates varied in the way that one would predict from the scheme we have been considering.

EXPERIMENTS REQUIRING SOME MODIFICATION OF THE SCHEME

A Potassium-Like Action of Extracellular Sodium

Several experiments have been reported showing that extracellular sodium can act as a poor substitute for extracellular potassium in catalysing the hydrolysis of phosphoenzyme (Glynn and Karlish, 1976; Lee and Blostein, 1980; Post *et al.* 1972; Beaugé and Glynn, 1979b). Because of this behaviour, the pump can catalyse a second kind of sodium:sodium exchange (Lee and Blostein, 1980; Cornelius and Skou, 1985). This differs from the classical sodium:sodium exchange in that it is slower, it occurs in the absence of ADP, it is accompanied by the hydrolysis of ATP, and it is electrogenic. All of these features are, of course, to be expected if extracellular sodium is acting merely as a poor substitute for potassium.

A Sodium-Like Action of Hydrogen Ions

Many years ago, Fujita *et al.* (1976) reported that, at low pH, NaI-treated pig brain microsomes showed phosphorylation by ATP, and potassium-dependent ATPase activity, in the absence of sodium ions. Similar findings were reported recently with a purified pig kidney enzyme (Hara *et al.* 1986), and Hara and Nakao (1986) have shown that the hydrolysis of ATP by enzyme incorporated into the membranes of artificial lipid vesicles is accompanied by the transport of hydrogen ions, presumably substituting as sodium ions.

The maximal rate of ATP hydrolysis at pH 5.6 was about 1/14 of the rate when sodium ions were present.

Direct Escape of Occluded Potassium to the Exterior

There is an important group of experiments by Karlish and Stein (1982), who studied rubidium fluxes across the membranes of artificial lipid vesicles containing incorporated Na,K-ATPase. They showed, surprisingly, that very small ouabain-sensitive fluxes could be demonstrated even in the complete absence of ATP and phosphate. It was tempting to suggest that these fluxes, which were very small, represented abnormal behaviour of enzyme that had been damaged in the incorporation process, but Kenney and Kaplan (1985) demonstrated similar fluxes in resealed red cell ghosts. The fluxes were very small, but their existence implies that occluded potassium ions can escape very slowly to the external medium even without phosphorylation by inorganic phosphate. That slow escape route probably explains a small discrepancy noted by Glynn *et al.* (1987) in experiments designed to compare the rate of release of occluded rubidium with the rate of the accompanying conformational change measured by stopped-flow fluorimetry.

Very recently, Kenney and Kaplan (1987; see also this volume) have reported some elegant experiments in which they studied the interchange of rubidium and lithium ions in resealed red cell ghosts. Since the rate of change of E_2 to E_1, which is very slow when the E_2 contains rubidium or potassium, is known to be fast when the E_2 contains lithium, they predicted that ATP would accelerate the exchange when rubidium was entering but not when lithium was entering; and that is what they found. They also found - though this is not predicted by the scheme - that though phosphate was required when rubidium was leaving the cells, it was not required when lithium was leaving the cells. It would seem to follow, that the direct escape from the occluded form to the external medium in the absence of phosphate, which is very slow for rubidium or potassium, is quite fast for lithium.

Simultaneous Binding of ATP and Orthophosphate

According to the scheme, as drawn, $E_2(K)$ can combine with ATP or orthophosphate but not both. If this were correct, potassium:potassium exchange should always be inhibited by excess ATP at any fixed level of orthophosphate, and by excess orthophosphate at any fixed level of ATP. In their studies of rubidium:rubidium exchange, however, Karlish *et al.* (1982) found that, though the expected inhibition occurred when the concentration of the fixed reactant (ATP or orthophosphate) was low, no inhibition was seen when the concentration of the fixed reactant was high. It seems to follow that ATP and orthophosphate must be able to bind simultaneously.

Transmembrane Allosteric Effects

In a series of experiments to study the activation by sodium or potassium ions of the different transport modes of Na,K-ATPase incorporated into the membranes of artificial lipid vesicles, Karlish and Stein (1985) found that the shape of the activation curves - the extent to which they were sigmoid - was affected in an unexpected way by the nature of the cations at the opposite surface. They concluded from their results that the binding of sodium ions to extracellular allosteric sites on the E_1 form of the dephosphoenzyme induces a subconformation (E_1^*) in which the cytoplasmic sodium-binding affinities of the transport sites are well separated whereas the potassium-binding affinities are close together. Conversely, in the absence of external sodium, the subconformation E_1 predominates, with cytoplasmic sodium-binding affinities close together and potassium-binding affinities spread out. They were also obliged to postulate that sodium ions bound to allosteric sites on the cytoplasmic surface exerted similar allosteric effects on cation binding to E_2 forms at the extracellular surface. They emphasize that, although they were forced to conclude that *allosteric* sodium sites at the extracellular surface co-exist with sodium or potassium *transport* sites at the cytoplasmic surface, there is nothing in their experiments incompatible with ping-pong kinetics. Transmembrane allosteric effects have recently been described also by Cornelius and Skou (this volume).

Occlusion of Sodium Without Phosphorylation

According to the scheme, phosphorylation of the enzyme by ATP is a prerequisite for the occlusion of sodium ions. Esmann and Skou (1985), however, found that sodium ions were occluded in enzyme treated with oligomycin, even in the complete absence of phosphorylation. Oligomycin is not, of course, normally present, so there is no reason to suppose that sodium ions can be trapped in the normal cycle other than by phosphorylation of the pump, but the result is important because it shows that whatever the negatively charged binding sites are that bind the sodium, they cannot include the *beta* aspartyl phosphate.

EXPERIMENTS REQUIRING MORE SERIOUS MODIFICATION OF THE SCHEME

A Third Phosphorylated Intermediate

There is now a substantial number of experiments done by several different groups of workers, mostly on bovine brain Na,K-ATPase, but also, to a lesser extent, on electric organ enzyme and kidney enzyme, that point to the existence of a third form of phosphoenzyme, intermediate between E_1P and E_2P. These experiments are of several different kinds.

The usual way of estimating E_2P is to expose enzyme briefly to labelled ATP in the presence of magnesium and sodium, and then, at zero time, to stop phosphorylation with excess cold ATP or CDTA, and at the same time to add a high concentration of potassium ions. There is a biphasic loss of phosphoenzyme, the rapid phase representing mostly the loss of E_2P, and the slow phase representing the loss of E_1P (either by direct hydrolysis or by conversion to E_2P and subsequent hydrolysis). By extrapolating back the slow phase and subtracting the intercept from the initial amount of phosphoenzyme, one gets an estimate of E_2P, the potassium-sensitive phosphoenzyme. A similar experiment, but with ADP added instead of potassium, gives an estimate of E_1P - the ADP-sensitive phosphoenzyme. Obviously, according to the scheme, the sum of E_1P and E_2P should equal the total amount of phosphoenzyme, and this has sometimes been found - see, for example, Fig 3 of Hara and Nakao (1981). Hara and Nakao found extremely good agreement over a wide range of sodium concentrations that led to great

change in the relative amounts of E_1P and E_2P. However, there are a number of other careful studies - by Klodos and Nørby (1979), Nørby *et al.* (1983) and Klodos and Nørby (1987), using ox brain enzyme; by Kuricki and Racker (1976) and Yoda and Yoda (1982, 1986, 1987a,b), using electric organ; and by Fukushima and Nakao (1981), using pig kidney enzyme but with Ca instead of Mg, - in which the sum E_1P plus E_2P exceeded the total amount of phosphoenzyme. This must mean that some of the phosphoenzyme - it is convenient to follow Yoda and Yoda in calling it E*P - was sensitive both to ADP and to potassium. (The sensitivity either to ADP or to potassium could, of course, have been the result of rapid conversion of E*P to E_1P or to E_2P. Both sensitivities cannot be explained in this way, however, since if E*P had been in rapid equilibrium with both forms, the disappearance of phosphoenzyme would not have been biphasic.)

The calculation of the amounts in the different compartments by back extrapolation depends on the assumption, in each case, that the rate constant describing the loss from the slow compartment was unchanging from the beginning of the experiment. This assumption is not justified for compartments in series, which, by hypothesis, these compartments were. Unfortunately, the error the assumption leads to is in the wrong direction to explain the discrepancy between EP_{total} and the sum of E_1P and E_2P.

The existence of an extra form of phosphoenzyme is obviously an awkward complication, and a good deal of effort has been spent in trying to explain the experimental facts in other ways - see especially Froehlich *et al.* (1983), Nørby (1985), Klodos and Nørby (1987). Despite these efforts, it looks as though the excess of E_1P plus E_2P over EP_{total} is real, at any rate under some conditions.

The notion that there are more than two forms of phosphoenzyme does not, anyway, rest solely on that discrepancy.

In the experiments of Nørby *et al.* (1983), the level of sodium was shown to affect the intercept of the slow phase after the addition of ADP, even when the sodium was added after phosphorylation had been stopped. This implies that sodium can convert *part* of the pool of ADP-insensitive phosphoenzyme rapidly into ADP-sensitive phosphoenzyme. At

first sight, it might appear that the change in intercept could be explained by supposing that sodium at moderately high levels accelerates the conversion of E_2P to E_1P, but such an acceleration would affect only the slope of the slow phase and not its intercept. The change in intercept implies that the ADP-*in*sensitive fraction is not homogeneous. This behaviour represent a further complication, since E*P is, by definition, ADP-sensitive, and it is not easy to see how its existence could explain the observation. Interestingly, though, the change in intercept was not seen in the experiments of Hara and Nakao (1981), where a wide range of sodium concentrations produced great changes in the slope of the slow phase. These were, of course, the experiments which showed good agreement between EP_{total} and the sum of E_1P and E_2P.

Further evidence for the existence of an intermediate form comes from experiments of Taniguchi *et al.* (1986). Using a stopped-flow technique, they measured simultaneously the light-scattering and the fluorescence changes following phosphorylation of enzyme labelled with the fluorescent probe *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide. The delay in the change in light scattering relative to the change in fluorescence suggested the presence of at least two species of ADP-sensitive phosphoenzyme, with low and high light scattering respectively, which appeared sequentially during the forward reaction.

Perhaps the most striking evidence for the existence of a third form of phosphoenzyme comes from the experiments of Lee and Fortes (1985). Enzyme was phosphorylated at different concentrations of sodium over a very wide range. At each sodium concentration, Lee and Fortes measured the amount of phosphoenzyme present, the rates of binding and of release of anthroyl ouabain, and the rate of hydrolysis of ATP. (Potassium was always absent.) The total amount of phosphoenzyme did not change, but at low sodium it would have been nearly all E_2P, and at the very high sodium concentrations it would have been all E_1P. The surprising and important finding is that the rate of binding of anthroyl ouabain showed a peak at about 500 mM sodium and so did the Na-ATPase activity. Lee and Fortes explain their results by supposing that the form that binds anthroyl ouabain fastest and is hydrolysed quickest is neither E_1P nor E_2P but an intermediate form - in other words E*P - probably with two sodium ions bound to it.

What determines whether E*P exists in detectable quantities or not? Generally, where investigators have found a discrepancy between the sum of E_1P and E_2P and the total amount of phosphoenzyme, the discrepancy has been greatest at intermediate sodium concentrations. This is seen very clearly in the experiments of Yoda and Yoda (1986), who suggest that whether the intermediate form E*P can be detected or not depends on the sodium concentration and on the relative affinities for sodium ions of the three phosphorylated forms. But experiments by Yoda and Yoda (1987a,b) on Na,K-ATPase incorporated into artificial lipid vesicles show that the interconversion of the three forms of phosphoenzyme is also sensitive to the temperature, the amount of cholesterol in the membrane, and the presence or absence of ionophores. In particular, a concentration of cholesterol of more than 35% seemed to interfere with the conversion of E*P to E_2P so that E*P accumulated. This effect was reversed by the addition of the ionophores monensin, nigericin and A23187. It is not clear how these ionophores were acting, since they are all carboxylic ionophores, which, ideally, catalyse ion exchange without carrying current. Nevertheless, they can all also work in minor modes in which they do carry current, and it is tempting to suppose that their action depended on preventing the build-up of an electrical potential across the vesicle membrane. This hypothesis fits in nicely with recent observations of Rephaeli *et al.* (1986). They used stopped-flow fluorimetry to follow the interconversion of E_1P and E_2P in FITC-labelled enzyme incorporated into artificial lipid vesicles. By adding suitable ionophores and arranging appropriate gradients of potassium ions or lithium ions, they were able to generate electrical potential differences across the membrane, and they found that the conversion of E_1P to E_2P was accelerated by making the inside of the vesicle - i.e. the originally extracellular surface of the pump - negative to the outside. The notion that the conversion of E_1P to E_2P involves a movement of positive charge in the direction cell interior to cell exterior is also supported by the observation of Borlinghaus *et al.* (1987) that pretreatment with chymotrypsin abolishes the transient current generated by the sudden addition of ATP to Na,K-ATPase-containing membrane fragments applied to black lipid membranes.

By varying the cholesterol content of their vesicles and using ionophores, Yoda and Yoda (1987b) were able to

choose conditions so that the predominant phosphoenzyme was E*P or E_2P, without altering the sodium concentration; this was kept fixed at 10 mM on both sides of the vesicle membrane. They found that the stoichiometry of the sodium fluxes and their relation to ATP hydrolysis differed markedly depending on which form of phosphoenzyme predominated. When the phosphoenzyme was mostly E_2P, the ratios (Na efflux):(Na influx):(ATP hydrolysed) were 2.8 : 1.8 : 1 (i.e. roughly 3 : 2 : 1), as if sodium ions were acting as a poor substitute for potassium ions. (Efflux and influx here refer not to exit from and entry to the vesicles, but to the original orientation of the pump in the cell membrane.) This behaviour is, of course, identical with the ATP-dependent sodium:sodium exchange first observed by Lee and Blostein (1980) in inside-out red cell vesicles. When the phosphoenzyme was mostly in the E*P form, however, the stoichiometry was 1.6 : 0.6 : 1 (i.e. it approached 1 : 0 : 1). To explain this, Yoda and Yoda suppose that the conversion of E_1P to E*P releases a single occluded sodium ion at the extracellular surface, and that hydrolysis of E*P releases the other two occluded sodium ions at the intracellular surface. The overall process represents an uncoupled efflux of sodium, but it differs from classical uncoupled sodium efflux, where (probably) three sodium ions are expelled per molecule of ATP hydrolysed (Glynn and Karlish, 1976). Of these three ions, presumably the first is released to the exterior by the conversion of E_1P to E^*P, and the remaining two are released to the exterior after conversion of E^*P to E_2P. It is important to appreciate that the reason Yoda and Yoda were able to observe an uncoupled sodium efflux with a stoichiometry approaching 1:0:1 is not just that the level of E*P was high, but that it was high because the abnormal cholesterol content of the membranes made the rate of conversion of E*P to E_2P low compared with the rates at which E*P was formed and hydrolysed. With normal membranes, at high (but not extremely high) extracellular sodium concentrations, the level of E*P will be high, but the enzyme will catalyse a sodium:sodium exchange accompanied by ATP hydrolysis with a stoichiometry approaching 3:2:1. This is because the rates of interconversion of E*P and E_2P are fast compared to the rate of hydrolysis of E*P.

Since the uncoupled sodium efflux with a stoichiometry of one sodium ion expelled per molecule of ATP hydrolysed is the fifth new flux mode I have mentioned, it may be

convenient to list all of the flux modes now known to exist - see Table 1.

TABLE 1. FLUXES THROUGH THE Na,K-ATPase

A. The classical flux modes
(for references see Glynn, 1985)

1. Normal forward cycle
2. Backward cycle
3. ADP-dependent Na-Na exchange
4. (Nucleotide+Pi)-dependent K-K exchange
5. Uncoupled Na efflux (3Na:ATP)
(For accompanying anions see Marin and Hoffman (this volume))

B. The more recently discovered flux modes

1. ATP-consuming Na-Na exchange
(Lee and Blostein, 1980)
2. Uncoupled K efflux (Sachs, 1986b)
3. K-K exchange in absence of nucleotides and Pi
(Karlish and Stein, 1982)
4. Uncoupled Na efflux (1Na:ATP)
(Yoda and Yoda, 1987b)
5. H-K exchange (Hara and Nakao, 1986)

The Kinetic Competence of E_1P

Let me now turn to the most awkward question of all. Can E_1P be converted into E_2P fast enough to account for the observed rate of hydrolysis of ATP. There does not seem to be any doubt that the reaction is fast enough when hydrolysis occurs in the absence of potassium, but there is considerable doubt about the adequacy of the rate of conversion when potassium is present.

The classical experiments were done by Mårdh in 1975, using ox brain enzyme and sophisticated rapid mixing devices at 20°C , and they suggested that there was no difficulty. In particular, Mårdh found that when enzyme was phosphorylated with labelled ATP in the presence of 10 mM-K, and excess cold ATP was then added, the phosphoenzyme

(presumably nearly all E_1P) disappeared with a rate constant of about 77 s^{-1}, which was fast enough. This estimate has, however, been criticised on a number of grounds - see especially Plesner *et al.* (1981). First, there are uncertainties about the baseline. However, examination of the appropriate curve in Fig 4 of Mårdh's paper suggests that over the first 25 msec., which is the crucial period, baseline errors are not likely to have been too serious. Secondly, the figure of 77 s^{-1} is much the highest of several estimates. However, it is also the one made under the conditions most appropriate for comparing with the overall reaction rate. The experiment in which Mårdh stopped phosphorylation with CDTA gave a lower rate, but there is evidence in Fig 3B of Mårdh's paper suggesting that CDTA may not have acted quickly enough. A third difficulty pointed out by Plesner *et al.* is that there is an apparent incompatibility in Mårdh's experiments. Consider the scheme:

$$\xleftarrow{k_{-1}} E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \xrightarrow{k_3}$$

In the steady state (ss) in the absence of potassium:

$$k_2.[E_1P] = (k_{-2}+k_3)[E_2P]$$

Therefore $[E_1P_{ss}]/[E_2P_{ss}] = (k_{-2}+k_3)/k_2$.

Because the rate of the slow phase after the addition of ATP plus ADP is the same as the rate of the slow phase after ATP alone, k_{-2} must be negligible compared with k_3.
It follows that:

$$[E_1P_{ss}]/[E_2P_{ss}] = k_3/k_2.$$

From measurements of the steady-state levels of E_1P and E_2P and the overall hydrolysis rate, Plesner *et al.* were therefore able to calculate k_2 from Mårdh's data in the absence of potassium. It worked out as 7-8 s^{-1}, i.e. about an order of magnitude less than the figure of 77 s^{-1} obtained in the presence of potassium. Either then, there is something wrong somewhere, or one has to suppose that the presence of potassium greatly accelerates the rate of conversion of E_1P to E_2P. That is possible, but it is not predicted by the scheme, and it runs counter to the findings

of Nørby *et al.* (1983) whose experiments on ox brain enzyme at 0°C suggested that potassium ions *slowed* the conversion. (A slowing effect of potassium ions on the conversion of E_1P to E_2P is also suggested by a recent observation of Klodos (this volume). She found that the simultaneous addition of ADP and potassium to phosphoenzyme prepared in low-sodium media led to a *greater* synthesis of ATP than the addition of ADP alone.) The experiments of Nørby *et al.* (1983) also showed that it was not possible to account for the hydrolysis rate at 0°C by multiplying the estimated amount of E_1P by the estimated rate constant for its conversion to E_2P.

The experiments just described all used ox brain enzyme. The rate of conversion of E_1P to E_2P has also been estimated in *Electrophorus* electric organ Na,K-ATPase, at 21°C, by Froehlich *et al.* (1983) and by Hobbs *et al.* (1985a). Two methods were used. In the first, ATP was added to enzyme in a medium containing both sodium and potassium, and the level of phosphoenzyme and the amount of inorganic phosphate liberated were measured over the first 100 msec. From computer fits to the observed initial burst in phosphate production and the rise and fall in the level of phosphoenzyme, Froehlich *et al.* calculated that the rate constant was about $300s^{-1}$. In similar experiments, Hobbs *et al.* obtained a figure of 500-2000 s^{-1}. The second method was to allow ATP to phosphorylate the enzyme in the absence of potassium for either 10 msec or 116 msec, and then to add potassium and EDTA, and follow the decrease in phosphoenzyme over the next 300 msec. The rationale of this experiment is that any E_2P present is rapidly dephosphorylated when potassium is added, and differences in the pattern of phosphoenzyme decay reflect differences in the proportions of E_1P and E_2P. In fact, although there was much more phosphoenzyme after 116 msec than after 10 msec, the decay curves were not very different. Computer simulation showed that, to be compatible with the results, the rate constant for the conversion of E_1P to E_2P must have been between 300 sec^{-1} and 2000 s^{-1}. Both methods therefore give estimates for the rate constant that would make E_1P kinetically competent. Furthermore, since the second method gives an estimate of the rate constant for the conversion of E_1P to E_2P in the absence of potassium, the agreement between the two methods suggests that in the electric organ Na,K-ATPase the presence of potassium ions does not greatly slow or accelerate the conversion.

A worrying feature of these experiments is that the disappearance of phosphoenzyme following the addition of potassium was triphasic, with a fast phase - presumably including E_2P (and ?E*P), a slow phase attributed to the existence of vesicles, and an intermediate phase that could not be identified with E_1P because its decay was too slow to be compatible with the amount of E_2P present. A further complication in the interpretation of the experiments of Hobbs *et al.* (1985a) - see also Hobbs et al. (1985b) - is that when ADP and EDTA were added simultaneously to phosphoenzyme formed in the absence of potassium, there was a rapid loss of about 20% of the phosphoenzyme, and this loss was accompanied by the appearance of inorganic phosphate in nearly stoichiometric amounts. This surprising finding cannot be explained simply as a species difference, since a similar effect was not seen by Yoda and Yoda (1986).

Given the disagreements between experimental findings by different workers, and the difficulties of interpretation, the question of the kinetic competence of E_1P as an intermediate in Na,K-ATPase activity must remain open.

Are Acid-Stable Phosphorylated Forms of the Enzyme Involved in the Normal Pump Cycle?

The unsatisfactory conclusion just reached makes it pertinent to ask the more fundamental question: are acid-stable phosphorylated forms of the enzyme involved in the normal cycle i.e. the cycle responsible for sodium:potassium exchange?

The answer is, almost certainly, yes. In the first place, we know that E_1P contains occluded sodium ions, and there is no reason to doubt that acid-stable phosphorylated forms of the enzyme are involved in sodium:sodium exchange and uncoupled sodium efflux. Secondly, the work on rubidium occlusion makes it almost certain that dephosphorylation of E_2P catalysed by external potassium ions leads, first, to the trapping of the ions and then, after a change in enzyme conformation, to their release at the intracellular surface. If the normal cycle does not involve acid-stable intermediates, we should therefore have to postulate that there are two very different sequences of events capable of carrying sodium ions outwards, and two very different

sequences of events capable of carrying potassium ions inwards. And in each case, the sequence with which we are familiar would be the sequence irrelevant to normal functioning.

Although the argument just given seems to me cogent, there is one crucial experiment which might be done and which, rather surprisingly, has not been done - though Mårdh (1975) came very close to doing it. Suppose we allow our Na,K-ATPase preparation to hydrolyse *unlabelled* ATP in the presence of both sodium and potassium, choosing concentrations in the physiological range, and allowing the hydrolysis to continue for a few cycles to ensure that the levels of all intermediate forms are steady. If we then add a quantity of *gamma*^{32}P-ATP *sufficiently small not to change the ATP concentration significantly*, the initial rate of incorporation of radioactivity into acid-stable phosphoenzyme should be not less than the overall hydrolysis rate, and probably equal to it. (It would exceed it only if ^{32}P were also incorporated into irrelevant groups.) The advantage of this experiment is that one does not have to know anything about the different forms of acid-stable phosphate or about their amounts. All that matters is the rate of ^{32}P incorporation. If the rate of incorporation is less than the rate of ATP hydrolysis, then at least some of the hydrolysis must be by a route that does not include acid-stable phosphorylated intermediates. All that is required is sophisticated mixing devices and a good enzyme preparation. It is, however, crucial that the concentration of ATP be not significantly changed by the addition of labelled ATP, since if it is - as it was, unfortunately, in Mårdh's experiment - one cannot assume that the levels of all intermediates will remain constant.

ORDERED RELEASE OF RUBIDIUM IONS FROM A SINGLE *ALPHA-BETA*-PROTOMER

I should like to finish this talk by describing very briefly some experiments that Dr D.E. Richards and I have been doing recently.

You will remember from Dr Forbush's talk that, when dephosphoenzyme containing occluded rubidium ions is treated with magnesium and inorganic phosphate, the enzyme is phosphorylated and there is a rapid release of either half

or all of the occluded rubidium depending on whether there are or are not free potassium ions (or ions of potassium congeners) in the medium (Glynn *et al.* 1985a; Forbush, 1985,1987). The reason for this very surprising finding is, we think, that the release of the occluded rubidium ions is ordered; if the first ions to be released are quickly (and repeatedly) replaced by ions from the medium the residual occluded ions will be released only slowly. One possibility is that the rapidly released ions represent one out of two ions in each *alpha beta* protomer. An alternative hypothesis is that the two halves of the dimeric enzyme are released in order, so that the rapidly released ions represent all of the ions in one of the *alpha beta* protomers of each dimeric enzyme molecule. In attempting to understand how the enzyme works it is important to know which of these alternatives is true. How can we decide between them?

Suppose we could knock out one *alpha beta* protomer in each dimeric enzyme molecule and then test the effect of adding phosphate and magnesium in conditions in which we should normally get 50% of the rubidium released rapidly. If each *alpha beta* protomer behaves independently, we should still get 50% of the rubidium released rapidly. If, however, release from the two halves of the dimer is normally ordered, then with one half inactivated we should expect to get either 100% release or no release - we cannot predict which. There is, of course, no way in which we can knock out one *alpha beta* protomer in each dimeric enzyme molecule, but if we treat the enzyme with some agent that attacks the *alpha beta* protomers randomly, and we stop the reaction when the Na,K-ATPase activity is reduced to half, we should have a preparation in which a quarter of the enzyme will have been completely inactivated, a quarter will be normal, and half will have one functioning *alpha beta* protomer. If we allow this enzyme to occlude rubidium ions through the route that involves phosphorylation and dephosphorylation, then only the functional protomers will contain occluded rubidium. Note that half of the occluded rubidium will be in the normal enzyme and half of the occluded rubidium will be in the enzyme with only a single functioning *alpha beta* protomer. Now consider what happens when we add magnesium and phosphate in conditions in which we expect a 50% release. If the independence hypothesis is correct, each functioning *alpha beta* protomer will release quickly one out of its two rubidium ions, so we should still find that half of the rubidium is released rapidly. On the

alternative, 'half-of-the-dimer' hypothesis however, we get a different prediction. The 25% of normal enzyme will release half of its occluded rubidium, which will amount to 25% of the total amount of occluded rubidium. The 50% of enzyme that has one functioning protomer will release either all or none of its occluded rubidium, in other words, 50% or none of the total occluded rubidium. So the total fraction of rubidium released, on the 'half-of-the-dimer' hypothesis, will be 75% (i.e. 25% plus 50%) or 25% (i.e. 25% plus nothing). We can get an even better discrimination between the two hypotheses by knocking out rather more than 50% of the *alpha beta* protomers, so that a greater fraction of the occluded rubidium is held in the enzyme with one functioning protomer, which behaves differently on the two hypotheses.

We have done the experiment in two ways, using either *alpha* chymotrypsin or fluorescein isothiocyanate to knock out half of the Na,K-ATPase activity. It is obviously crucial to the experiment that *alpha beta* protomers with no ATPase activity do not occlude rubidium in the circumstances of the experiment. We therefore checked that progressive treatment with each of the reagents gave a proportional reduction in ATPase activity and rubidium occlusion. When we came to do the experiment proper, we found that the fraction of occluded rubidium lost quickly from enzyme whose Na,K-ATPase activity had been inhibited to 50% or more (with either reagent) was still close to 50% and very significantly different from the fractional losses expected on the 'half-of-the-dimer' hypothesis. In other words, it is the release of the two rubidium ions in each *alpha beta* protomer that is ordered, not the emptying of the two halves of the dimer.

The experiment with the chymotrypsin-treated enzyme is not absolutely unequivocal, since one could suppose that, when chymotrypsin attacks the enzyme, the breaking of the polypeptide chain in one protomer makes the other protomer extremely vulnerable, so that the two protomers are, as it were, picked off in pairs. The experiment with FITC-treated enzyme is not open to this kind of objection, however, as P.A.G. Fortes (personal communication) has shown that there is strict proportionality between FITC binding and Na,K-ATPase inhibition, with complete inhibition occurring when one FITC molecule is bound per phosphorylation site.

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SYMMETRIC ACTIVE TRANSPORT IN CHOLATE-DIALYSED LIPOSOMES CONTAINING RANDOMLY ORIENTED SODIUM PUMPS

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INTRODUCTION

Despite the recent determination of the amino acid sequence of the α and β subunits of the Na,K-ATPase protein in several laboratories (Shull et al., 1988), the tight link between the extracellular receptor and the transmembrane active transport catalyzed by intracellular ATP is still poorly understood. A sided model-system containing purified Na,K-ATPase molecules performing active transport, is a prerequisite to connect specific structural modifications to alterations of the active transport and of the transport-receptor interaction.

We have now designed such a model-system based on the previous extensive analysis of numerous cholate-dialysed Na,K-ATPase-liposome preparations by electron microscopy that determined the liposome size distribution, the average entrapped volume per liposome, the number of Na,K-ATPase molecules per liposome and their orientation (Anner et al., 1984; Anner and Moosmayer, 1985).

On the average, four co-reconstituted pumps, i.e. two inside-out oriented (i-s-o) and two right-side-oriented (r-s-o) pumps perform active transport per liposome of 100 nm diameter, by adding first internal ATP and external RbCl and then, when sufficient internal RbCl has accumulated to drive the i-s-o pumps, external ouabain (to block the r-s-o pumps) and external ATP (to activate the i-s-o pumps).

Exploiting this bifunctional model, we noticed, first, that the transmembrane Rb-gradient did not influence the pump-rate, and, second, we confirmed the pump orientation in freeze fractured liposomes.

RESULTS

The equal intramembrane particle density on concave and convex faces of freeze-fractured liposomes (Table 1) indicates a symmetric orientation of the reconstituted Na,K-ATPase, provided that the fracture plane goes asymmetrically around the pump.

TABLE 1 . Symmetric distribution of intramembrane particles in cholate-dialysed freeze-fractured liposomes

$$\frac{\text{IMP density (concave surface)}}{\text{IMP density (convex surface)}} = 1.022 \pm 0.052^{*}$$

*Value is mean ± S.D.; the number of intramembrane particles of 250 concave and 250 convex liposome faces of 17 different preparations were counted.

A putative mechanism by which the cholate-dialysis procedure changes the original asymmetric orientation of the enzyme to a symmetric one is outlined in Fig. 1. Another possibility might be that artificial $\alpha_2\beta_2$ dimers are formed by the enzyme purification procedure, where the intracellular part of one $\alpha\beta$ monomer associates with the extracellular part of another $\alpha\beta$ (Zampighi et al., 1986), leading to units which perform active transport in both directions if the $\alpha\beta$ complex is the minimal pump unit.

The ratio of the average water space entrapped by the standardized Na,K-ATPase-liposomes preparation to the

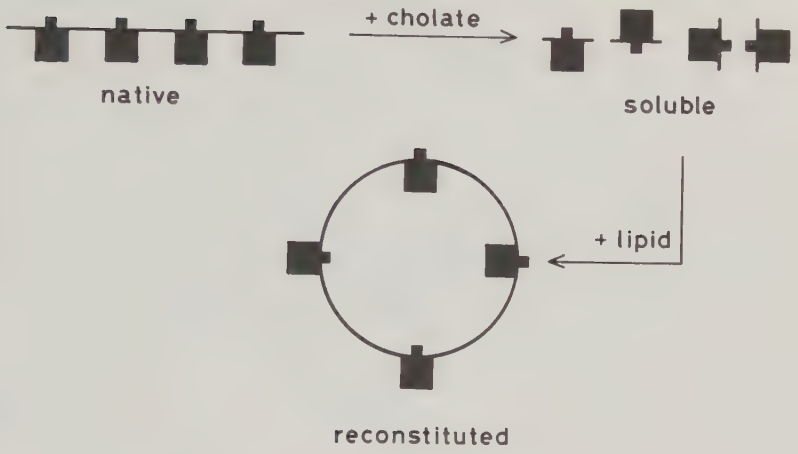

Figure 1. Passage from asymmetric to symmetric Na,K-ATPase orientation by solubilization of 5 μM purified Na,K-ATPase protein (assuming a mol. wt. of 300 000 dalton for the $\alpha_2\beta_2$ unit) in 23 mM sodium cholate, 50 mM Na_2ATP, 5 mM $MgCl_2$, 30 mM histidine, 1 mM Tris-EDTA, pH 7.10, 12 mM dioleoyl-phosphatidylcholine and accelerated removal of the cholate by dialysis at 0°C in the presence of cholestyramine resin (Rey et al., 1987).

average volume of a single liposome yields the average number of liposomes per volume, which is equal to the liposome density illustrated in Fig. 2. As a consequence, the number of substrate molecules entrapped per liposome as well as the number of external molecules and ions in the surrounding solution can be calculated during both resting state and active transport.

In theory, at identical bilateral pump-ligand concentrations and with a symmetric pump orientation, the active transport should proceed at equal rate in both directions. However, the Rb-extrusion rate was consistently faster than the uptake rate, even at saturating RbCl concentrations (Rey et al., 1987). Finally, we discovered that ATP concentrations above 5 mM lowered the pump turnover (Fig. 4).

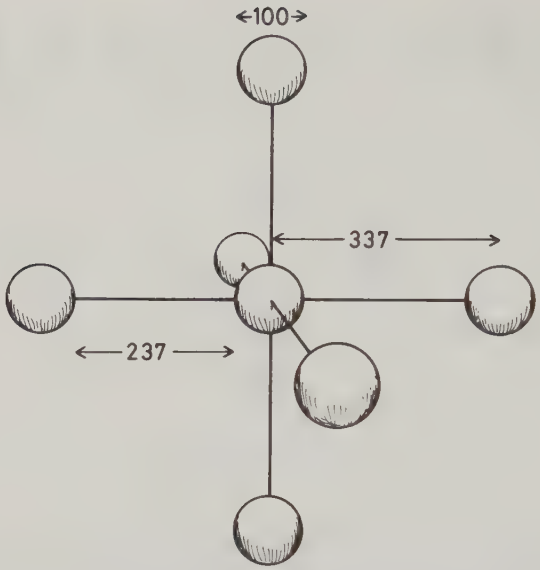

Figure 2. Calculated density of the standardized Na,K-ATPase-liposome suspension prepared as described in Legend of Fig. 1. Values are nm. The ratio of the entrapped space to the external space is 1%. Thus, 1 ml suspension contains 2.6 x 10^{13} liposomes representing an external surface of 8 230 cm^2.

Fig. 3 shows the principle of the bifunctional liposomes. Excess Na and Mg ions and ATP are present on both sides of the membrane so that Rb ions are the sole regulator of the pump rate.

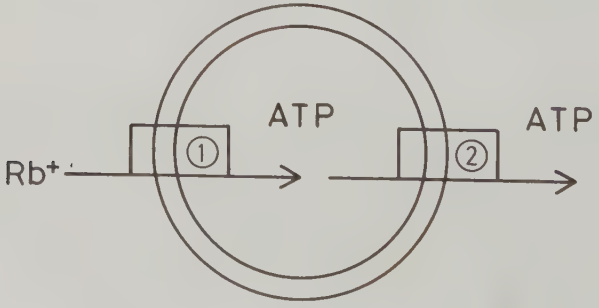

Figure 3. After cholate-dialysis and removal of the external ATP (see Legend of Fig. 1), an average 100 nm liposome contains about 10 000 ATP (50 mM) molecules, 40 000 Na ions (200 mM), and 1000 Mg ions (5 mM). The r-s-o pump population (1) is activated by the addition of 5 to 2 000 μM external RbCl. When the liposomes have accumulated the desired concentration of Rb ions (usually an inside-out Rb gradient ranging between 1 and 70), 100 μM ouabain are added externally, together with 10 to 50 mM ATP to block the r-s-o and to activate the i-s-o pumps (2) which then extrude the accumulated Rb-ions.

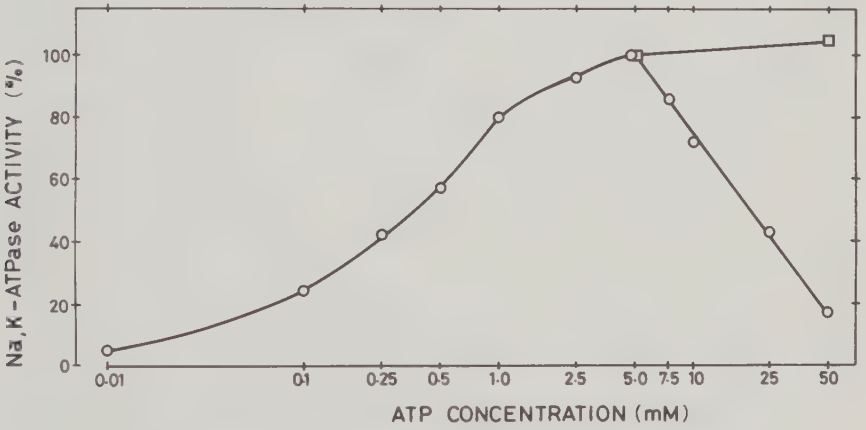

Figure 4. Inhibition of enzyme turnover by high ATP concentrations (○). Na,K-ATPase activity was measured at 37°C by the enzyme-linked (PK/LDH - NADH) assay at increasing ATP concentrations. Control experiments ruled out that the inhibition was due to increased ionic strength by NaCl (□) or Tris (not shown). The enzyme turnover was 3.9 ± 0.3 (n = 4) times lower at 50 mM than at 10 mM ATP.

Fig. 5 shows the typical triangular curves obtained by successive activation of r-s-o and i-s-o pumps. The 50 mM ATP-containing Na,K-ATPase-liposomes were incubated at 25°C in the presence of external RbCl to drive Rb accumulation via the r-s-o pumps (Fig. 5). The linear phase was determined by measuring the ^{86}Rb-uptake at min intervals; external ATP was added to extrude the captured ^{86}Rb, via

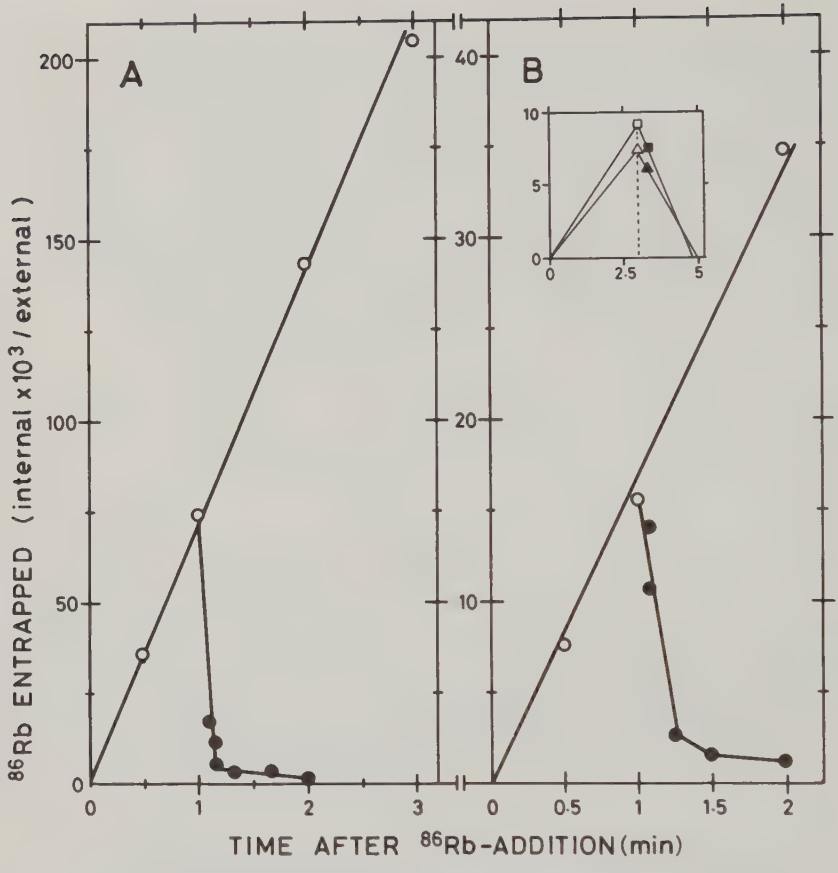

Figure 5. Successive active Rb-transport by co-reconstituted r-s-o and i-s-o Na,K-ATPase. At time 0, 10 µM (A) or 1 mM (B) external ^{86}RbCl are added to aliquots of the ATP-containing liposomes to start the turnover of the r-s-o pumps; 10 mM (○) or 50 mM (□, △) external ATP and 100 µM ouabain are added to activate the i-s-o oriented pumps (●, ■,) and to inhibit the r-s-o oriented pumps (○, □, △). In the absence of external ATP, the r-s-o pumps continue to accumulate Rb ions (○). By adding 10 mM external ATP instead of 50 mM, the i-s-o pumps are less inhibited (inset). The internal RbCl concentration is calculated from the 10 µl water space entrapped by 1 ml standardized liposome preparations, i.e., ^{86}Rb-entrapment above 1 % indicates inside-out Rb-gradients. Each experimental point represents 1 - 2 measurements in a typical preparation.

the i-s-o pumps. The ratio of the ^{86}Rb-extrusion-rate to the uptake-rate is determined by dividing the descending by the ascending slope, at both suboptimal and saturating (external and internal) RbCl concentrations.

CONCLUSIONS AND PERSPECTIVES

Co-reconstituted i-s-o and r-s-o oriented Na,K-ATPase molecules in liposomes are activated successively by asymmetric addition of ATP to the internal and external liposome copartment. In the presence of mirror-symmetrical conditions, i.e. identical and saturating bilateral pump-ligand concentrations, the transport rates of the two pump populations are about equal, indicating that the random distribution of intramembrane particles, seen in freeze-fractured preparations, corresponds to the actual pump orientation. This observation provides the first functional confirmation of the random distribution of intramembrane particles in Na,K-ATPase-liposomes.

The model, in which the extra- and intracellular sides of actively transporting Na,K-ATPase molecules are successively exposed at the exterior surface of liposomes is a helpful tool for studying the structure-function relationship of Na,K-ATPase.

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NA,K-ATPASE IN ARTIFICIAL LIPID VESICLES: POTENTIAL DEPENDENT TRANSPORT RATES INVESTIGATED BY A FLUORESCENCE METHOD

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INTRODUCTION

The Na,K-ATPase in the plasma membrane of animal cells transports sodium ions outward and potassium ions inward (Skou, 1975; Glynn, 1985). Since under physiological conditions the sodium flux is larger than the potassium flux, the pump is electrogenic. Therefore at least some of the partial reactions carry charge in the electrical field generated by the membrane potential. For thermodymanic reasons these partial reactions are affected by the membrane potential and should influence in an appropriate potential range the turnover rate of the pump (De Weer et al., 1988). By investigating the voltage dependence of the pump current information on the transport mechanism may be obtained (Chapman et al., 1983, Blaustein and Lieberman, 1984; Läuger and Apell, 1986).

MATERIALS AND METHODS

Lipid vesicles were prepared from synthetic dioleoylphosphatidylcholine as described previously by a dialysis method producing homogeneous, unilamellar vesicles with an average outer diamter of 72 nm (Apell et al., 1985). Na,K-ATPase was prepared from outer medulla of rabbit kidneys using procedure C of Jørgensen (Jørgensen, 1974), as described by Anner et al. (1984). Reconstituted vesicles containing Na,K-ATPase were prepared by cholate removement in "buffer H" containing 30 mM imidazole, 1 mM L-cystein, 1 mM EDTA, 5 mM $MgSO_4$ and various amounts of Na_2SO_4 and K_2SO_4. The experiments were performed as described before (Apell and Bersch, 1987).

The effect of membrane potential on the fluorescence of oxonol VI can be explained by the following mechanism (Apell and Bersch, 1987): A partition equilibrium of dye between membrane and water was found to exist with a partition coefficient $\gamma \approx 19000$. If f_w and f_l are the contributions of one mole dye in the water and in the lipid to the observed fluorescence, the total fluorescence intensity F can be represented by

$$F = n_w f_w + n_l f_l = n\,(f_w V_w + \gamma f_l V_l)/(V_w + \gamma V_l) \qquad (1)$$

where n_w and n_l are the amounts of dye in the water and lipid phase and $n = n_w + n_l$ is the total amount of dye in the fluorescence cuvette. V_w and V_l are the volumes of the water and lipid phases. F is referred to the fluorescence intensity measured with a fluorescence standard and is expressed in dimensionless units, so that f_w and f_l have the dimension mol^{-1}.

In the presence of an inside-positive membrane potential, the negatively charged dye accumulates in the intravesicular aqueous space according to a Nernst equilibrium

$$U_m = RT/F \ln (c^o/c^i) \qquad (2)$$

U_m is the membrane potential, c^o and c^i are the extra- and intravesicular dye concentrations. The Nernst equilibrium leads to an increased adsorption of dye to the inner lipid monolayer and to a concomitant increase of fluorescence. The fluorescence change can be calibrated as a function of transmembrane voltage by generating a potassium diffusion potential in the presence of valinomycin. Due to the restricted intravesicular space and due to the presence of a charged permeable dye the accurate membrane potential has to be corrected with respect to the Nernst equation. An analysis of experimental results exhibited that only the partition coefficient γ of Eq.1 is voltage dependent, whereas the specific fluorescence f_l remains unaffected.

The voltage dependence of the apparent partition coefficient γ can be quantitatively described by a three-capacitor model in which the dye is assumed to bind to adsorption planes located on the hydrocarbon side of the membrane-solution interface. This model finally allows a correct description of measured calibration curves.

RESULTS AND DISCUSSION

Reconstituted Na,K-ATPase vesicles formed by detergent dialysis contain pump molecules with either orientation (Anner, 1985a; Anner, 1985b). By adding ATP to the extravesicular medium only those pumps with outside-facing ATP-binding site become activated. Since the Na,K-ATPase normally transports 3 Na^+ ions outward and 2 K^+ ions inward (Glynn, 1985), activation of inside-out-oriented pumps should generate an inside-positive membrane potential in the vesicle (Apell et al., 1985). An experiment demonstrating this electrogenic effect of the pump is shown in Fig. 1. After addition of 2.5 mM ATP to the vesicle suspension at a temperature of 11.2°C, the fluorescence starts to increase, reaching a plateau after about 80 s. From the plateau value of $\Delta F/F_o$ (≈ 0.8) and the calibration curve for 35 μg/ml lipid, the maximum voltage is estimated to be about 90 mV (inside positive). When vanadate, a potent inhibitor of the Na,K-pump (Glynn, 1985) is added to the medium, the signal declines, approaching an asymptotic value with a time constant of about 400 s.

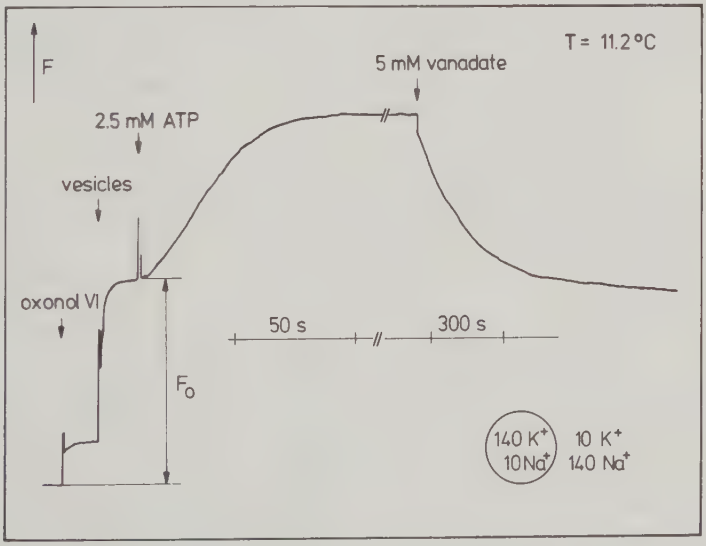

Figure 1. Membrane potential generated by the Na,K-pump. The average number of pump molecules with the ATP-binding site facing outward was determined to be n ≈ 1. 52 nM oxonol VI, 35 μg/ml lipid, 2.5 mM ATP and 5 mM vanadate were added successively. F is the fluorescence intensity.

The decline of fluorescence intensity is likely to result from a decay of membrane voltage via the leakage conductance of the vesicle membrane. If this is true, then the time constant τ should be equal to C_m/G_m, where C_m and G_m are the specific capacitance and the specific conductance of the membrane, respectively. With $\tau \approx 200$ s and $C_m \approx 1\ \mu F/cm^2$, G_m becomes about 5 nS/cm^2, which is a reasonable value for a lipid bilayer. From the initial slope of $\Delta F/F_o$ versus time t in Fig.1, the initial rate of voltage change can be estimated to be $dU/dt \approx 1.75$ mV s^{-1} which must be equal to ne_ov_o/AC_m where n is the average number of (outward oriented) pump molecules per vesicle, e_o the elementary charge, v_o the initial turnover rate and A the average area of the vesicle membrane. In the vesicle preparation used for the experiment of Fig. 1, n was determined from the potassium-flux rate (Apell et al., 1985) to be about 1. This yields, with $A \approx 0.025\ \mu m^2$ and $e_o \approx 1.6 \cdot 10^{-19}$ C, an initial turnover rate of $v_o = AC_m(dU/dt)/ne_o \approx 2.8\ s^{-1}$. On the other hand, if v is the turnover rate in the quasistationary state in which the pump current is compensated by a backflow of charge through the membrane conductance C_m, the relation $ne_ov = AG_mU_s$ holds where $U_s \approx 90$ mV is the stationary voltage. This gives $v = 0.058\ s^{-1}$. Thus, the turnover rate v in the quasistationary state is about fifty times smaller than the initial turnover rate v_o. The difference between v and v_o is likely to reflect the voltage dependence of pumping rate (Läuger and Apell, 1986).

The pump induced potential can formally be described as the charging of a capacitor. Therefore a relation exists between the current across the vesicle membrane and the change of membrane potential

$$I = AC_m\ dU/dt \qquad (4)$$

and the current I is the sum of two oppositely directed components

$$I = I_{pump} + I_{leak} = n_p e_o \cdot v(U) - AG_m \cdot U \qquad (5)$$

with n_p the number of pump per vesicle and v(U) the voltage-dependent pumping rate (the other symbols as above). From Eqs. 4 and 5 the voltage dependence of the current of a single pump $v(U) \cdot e_o$ or the pumping rate v(U) can be determined:

$$e_o \cdot v(U) = (C_m \cdot dU/dt + G_m \cdot U)\, A/n_p \qquad (6)$$

Since the calibration curve for fluorescence signals is known, at every point of the time course after the activation of the pump, the membrane potential U can be determined from the size of the fluorescence signal and the time derivative dU/dt from the slope of the fluorescence signal. In Fig. 2 such an analysis has been performed for a set of experiments in the temperature range between 14 and 20°C. The temperature dependence of $v(U = 0) = v_o$ leads to an activation energy of 160 kJ/mol and is in accordance with previously published data (Apell and Bersch, 1987). This analysis used values of A and n_p which were determined to be the average values for this vesicle preparation (Apell et al., 1985).

It is known that vesicles differ in radius and number of transport proteins (Anner et al., 1984); therefore the surface of the vesicles A and the number of pumping cannot be kept constant. An exact treatment of this problem (Apell and Läuger, 1986) shows that an analytical solution is not possible. Therefore computer simulations have been performed according to Apell and Läuger (1986) starting with v(U)-curves determined by the assumption of homogeneous vesicles. The simulation

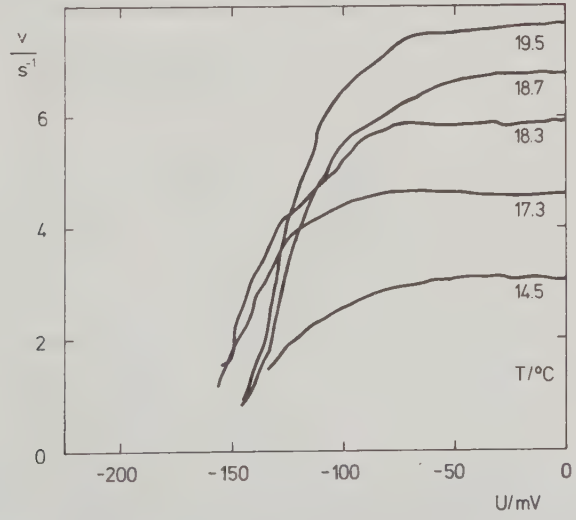

Figure 2. Voltage dependence of the pumping rate v at different temperatures determined according to Eq.6 with $A = 2.3 \cdot 10^{-10}$ cm^2 and n_p=5.2.

allows different sets of v(U)-curves which would be accepted as fit. Fig. 3a shows an experiment and a possible simulation and Fig. 3b the appertaining analysis according to Eq.6 and the confidence range of simulation for the inhomogeneous

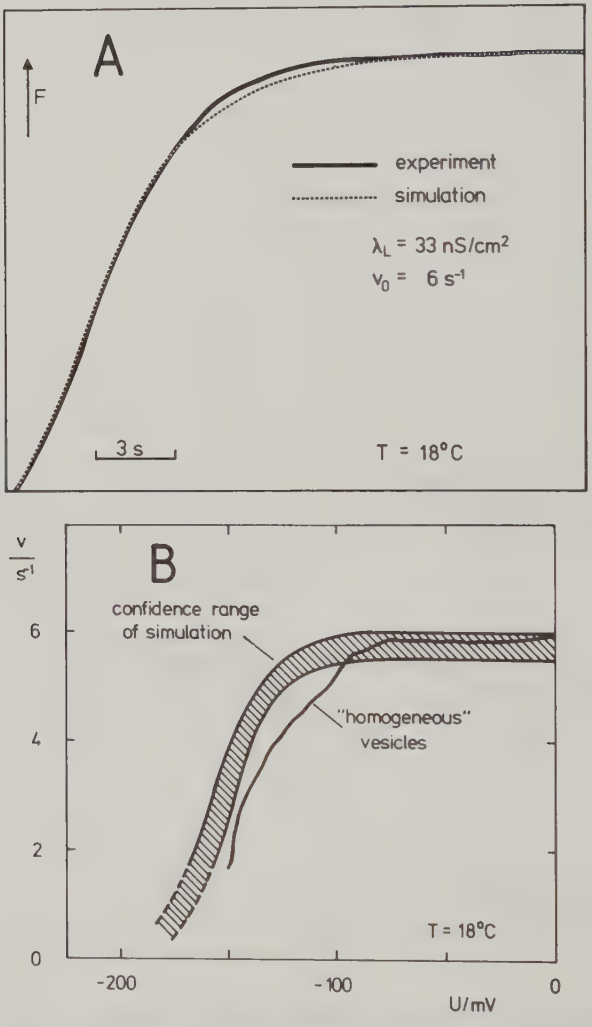

Figure 3. a) Increase of membrane potential generated by the Na,K-ATPase, detected by oxonol VI fluorescence, compared with a simulation according to Apell and Läuger (1986). b) Appertaining v(U)-curve: solid line calculated according to Eq.6, hatched region determined as range of v(U)-curves which give acceptable fits.

vesicle preparation. The basis of the hatched region are 5 experiments at 18°C. The simulations exhibited that the form of the v(U)-curve below a voltage of -150 mV has no significant influence onto the calculated fluorescence signal. An explanation can be seen in the flattening calibration curve of oxonol VI (Apell and Bersch, 1987).

Predictions about the form of the v(U)-curve in the positive potential range were made by investigations with an indocyanine dye in a similar system as described in this paper, where the experiments could be described precisely with a voltage independent pumping rate v in the range between 0 and +60 mV (Apell et al., 1985). These findings are in principal agreement with the predicted current voltage curve with low-resistance access channels and two or three negatively charged ion-binding sites (Läuger and Apell, 1986). Karlish el al. (1985) found clear evidence supporting two negatively charged ion binding sites.

Compared with recently published I-U-curves (Gadsby et al., 1985; Lafaire and Schwarz, 1985) measured in native tissues an approximal agreement in shape was found only with Gadsby's data. Possible sources of the evident differences may be found in symmetrical lipid composition in the reconstituted system and/or in the presence of other potential dependent transport proteins in the native system.

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FAST CHARGE TRANSLOCATIONS ASSOCIATED WITH PARTIAL REACTIONS OF NA,K-ATPASE INDUCED BY ATP CONCENTRATION-JUMP

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INTRODUCTION

An important aspect of the electrogenic nature of the pump is the possibility of obtaining mechanistic information by studying the electric current generated by the pump. Currents associated with active transport of Na^+ and K^+ have been investigated so far mainly in the steady state of the pump; independent information on the kinetic parameters of the pumping cycle can be obtained by studying pump current in the nonstationary state after a perturbation of the transport system. In the following, experiments are described in which nonstationary pump currents are induced by a sudden change of ATP concentration.

METHODS

For the measurement of transient current signals, flat membrane fragments rich in Na,K-ATPase are bound to a planar lipid bilayer acting as a capacitive electrode. The method of capacitive coupling has been first used for studying fast charge translocations in membranes by Drachev et al. 1974. The membrane preparation (Jørgensen, 1974) consists of flat membrane sheets of 0.2-1 μm in diameter containing oriented Na_2K-ATPase molecules with a density of several thousand per μm^2 (Deguchi et al., 1977). In the aqueous phase which is in contact with the bound membrane sheets, ATP is released within milliseconds from an inactive, photolabile derivative ("caged ATP") by an intense flash of light (Kaplan et al., 1978, McCray et al., 1980). The teflon-cell, which was used

to carry out the bilayer experiments (Borlinghaus et al., 1987) consisted of two compartments, separated by a thin septum with a hole (1.3 mm diameter) in which the bilayer could be prepared. In the trans-compartiment a quartz-window was mounted through which the UV-light could be focussed on the membrane. The solutions on either side of the lipid membranes were connected to the external measuring circuit via black agar bridges and Ag/AgCl-electrodes. For current measurements under short-circuit conditions the signal was amplified with a Keithley Mod. 427 current amplifier. For measuring voltage signals, a Mod. 3528 Burr-Brown amplifier was used. Normal electrolyte conditions were: 20 mM $MgCl_2$, 20 mM Dithiothreitol, 50 mM Tris, pH 7.0, various concentrations of NaCl and caged ATP. ATP was liberated by an UV light flash generated by a Xenon-flash tube Mod. 5M-3 from EG&G Salem.

RESULTS

After addition of Na,K-ATPase membranes to the cis-compartment and smooth stirring, the sheets associate spontaneously

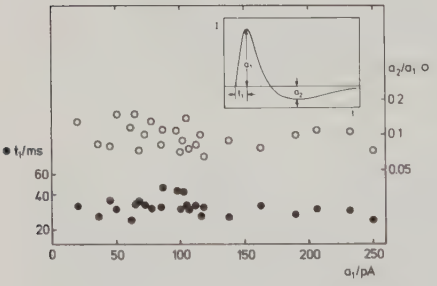

Figure 1. Dependence of t_1 and a_2/a_1 on the positive peak amplitude a_1. Inset: typical shape of measured current I(t).

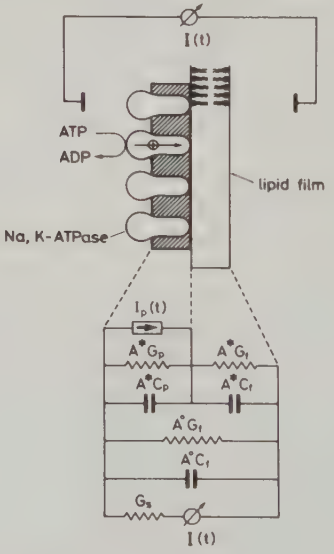

Figure 2. Equivalent circuit of the compound membrane system.

with the bilayer. The response of an UV-flash in presence of caged ATP, protein, Mg^{2+} and Na^{+} is shown in the inset of Fig. 1. The current rises fast and declines slowly to a negative value and then goes to zero. The maximum-amplitude both of the positive and the negative transient increases within about half an hour, then decreases slowly within several hours. The shape of the transients however, is not changed as shown in Fig. 1: The quotient of the two maximum amplitudes and the time to peak t_1 is not a function of the positive peak amplitude, so we decided to use kinetic parameters which are independent from the age of the membrane as empiric parameters to describe the signals.

The compound membrane system, consisting of a black lipid film with adsorbed Na,K-ATPase fragments may be represented by the equivalent circuit shown in Fig. 2. G_p and C_p are the conductance and the capacitance per unit area of the membrane fragments; G_f and C_f refer to the black film. (G_p also accounts for conductive pathways parallel to the bilayer). A^o and A^* are the areas of the uncovered and covered part of the film. $1/G_s$ is the series resistance of the circuit. The ma-

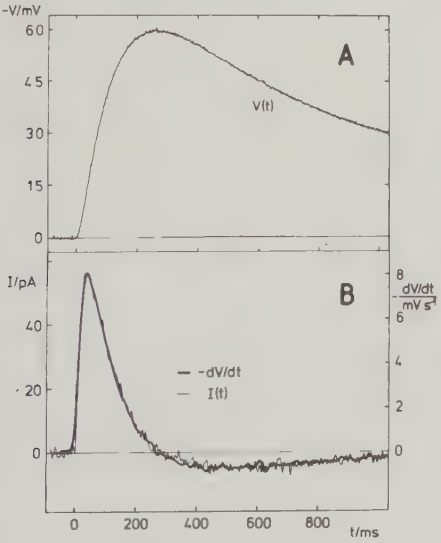

Figure 3. A: Voltage-transient V(t). B: Time-derivative of -V(t) and current-transient I(t) drawn to scale.

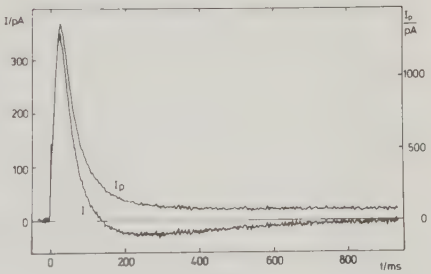

Figure 4. Current-transient I(t) and intrinsic pump current $I_p(t)$, calculated from I(t) by applying Eq. (1).

thematical analysis of the circuit results in an equation for an arbitrary intrinsic pump-current I_p as a function of C_p, C_f, τ_v and the measured current I (Borlinghaus et al., 1987):

$$I_p(t) = 1 + \frac{C_p}{C_f}\left[I(t) + \frac{1}{\tau_v}\int_o^t I(t)dt\right] \qquad (1)$$

In the absence of K^+ τ_v can be determined by an open-circuit voltage measurement (Fig. 3A). The declining of V after reaching a maximum is interpreted as flow of Na-ions out of the gap between sheets and bilayer and thus contains the information of the decharging of the sheet-capacitor with an RC-time τ_v. The analysis predicts a proportionality between the current I(t) and the time derivative of the voltage V(t). As a consistency-test we compared I(t) and -dV(t)/dt and found a very good verification (Fig. 3B). The result of applying Eq. (1) on a measured current-transient is shown in Fig. 4. $I_p(t)$ denotes the calculated intrinsic pump-current. After a fast transient we found a stationary current. The reason could be a slow steady state turnover-rate limited by the spontaneous dephosphorylation of the protein; this is al-

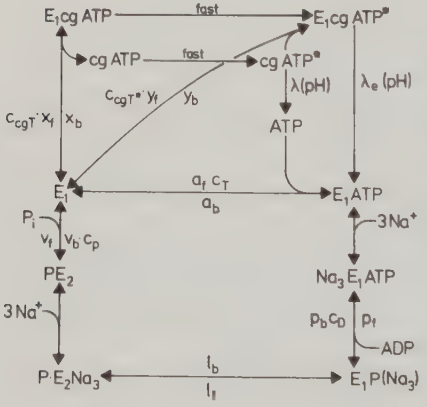

Figure 5. Reaction scheme of the pump-molecule in absence of K^+.

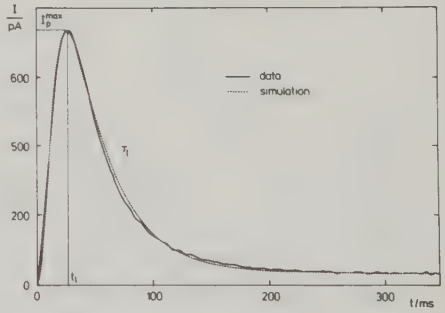

Figure 6. Comparison of measured and calculated $I_p(t)$.

so suggested by the results of double-flash-experiments (see below).

From studies of McCray et al. 1980 it is known that the rate limiting step in the photochemical release of ATP is catalyzed by H^+ with a rate constant $\lambda \approx (2.2 \cdot 10^9 M^{-1} sec^{-1}) \cdot [H^+]$ at 22°C. The time course of the flash-induced current signal was found to depend on the pH of the solution. The time to peak t_1 is pH-insensitive below pH 6 but strongly increases with pH above pH 6.5. t_1 is considerably larger than $1/\lambda$ below pH 7, but becomes comparable to $1/\lambda$ at pH > 8. This indicates that at high pH the rise time of the current signal is limited by the rate of ATP release. Furthermore, it is obvious that at pH < 7 the time for the current signal to reach its peak value is much shorter than the steady-state cycle time of the pump under optimal conditions (Apell et al., 1987).

The factors influencing the time course of the current signal were further investigated by double-flash experiments. When a second flash is given before or shortly after the current maximum an increase in current amplitude is observed in

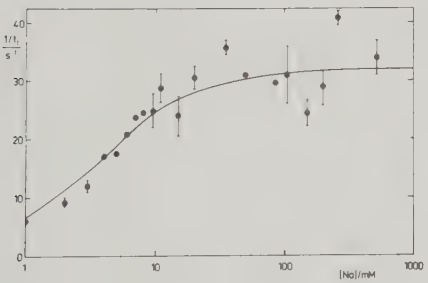

Figure 7. Dependence of $1/t_1$ on [Na].

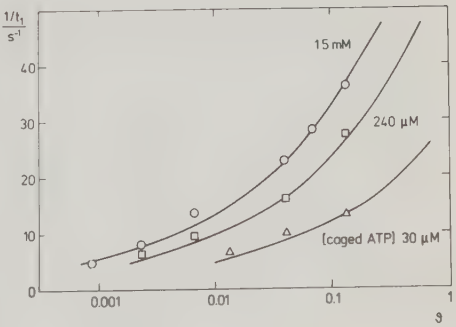

Figure 8. Dependence of $1/t_1$ on [caged ATP] and ϑ.

agreement with the fact that only about 10% of caged ATP is photolyzed in a single flash. When the waiting-time Δt between the two flashes is increased, the current increment becomes smaller and is about zero for 1 sec $< \Delta t <$ 2 min. The duration of this refractory state depends on the rate by which ATP is eliminated from the solution by apyrase VI, an enzyme that splits ATP and ADP to AMP and P_i (Liébecq, 1963). The observation that apyrase VI speeds up the recovery time is explained by the assumption, that at finite ATP concentration the pump is predominantly in the phosphorylated form. From the results of the double-flash experiments in the absence of apyrase it may be concluded that the decline of the current signal is not caused by depletion of ATP.

To test whether the data fit the Albers-Post-model, we calculated the pump-current $I_p(t)$ by numerical solution of the differential-equations, which describe the reaction-cycle. Fig. 5 shows the model we used. The lower part is the Na-part of Albers-Post-scheme, the phosphoenzyme is assumed to decay spontaneously. The upper part contains the reactions of ATP and caged ATP, which compete for the ATP-binding-site. The caged compound can split both soluted and bound to the

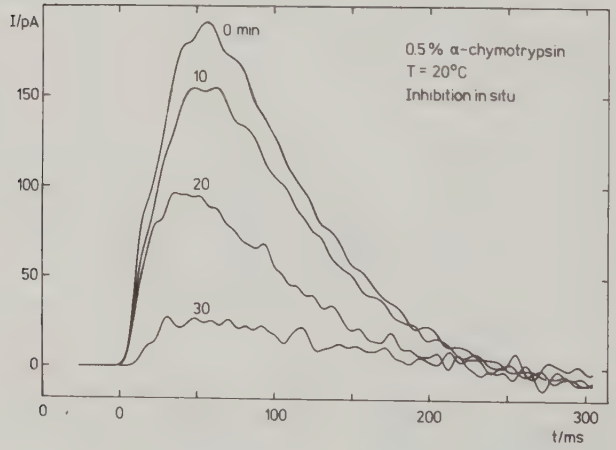

Figure 9. Development of I(t) after addition of chymotrypsin to the bound Na,K-ATPase.

enzyme. The rate-constants we used to calculate I_p were taken from the literature (see Läuger and Apell, 1986). In Fig. 6 measured and calculated pump currents are compared. To get acceptable fits only slight variations of the rate constants were necessary.

To test the validity of the model, we changed external parameters. The results of variation of the sodium concentration [Na] is shown in Fig. 7. The dots are mean values of $1/t_1$, averaged from several membranes. The line shows the evaluation of $1/t_1$ when $I_p(t)$ is calculated with fixed rate constants and only [Na] is changed.

Similar experiments were done with different ATP concentrations. The ATP-concentration can be controlled either by the caged-ATP concentration or by the yield ϑ of splitted ATP, which is a function of light intensity. The measurements coincide very well with the prediction (Fig. 8).

To decide which step in the Na-translocation is electrogenic, experiments were performed with chymotrypsin-treated Na,K-ATPase, which is known only to occlude Na and not to undergo the conformational change $E_1P \rightarrow E_2P$ (Glynn et al., 1984). If chymotrypsin is added after the sheets have bound to the target-bilayer, the current decreases to zero within about 1 hour (Fig. 9), but no significant change in the shape of the trace can be observed. This is interpreted as a break down of the number of active pump-molecules and indicates that no electrogenic step occurs when the deocclusion is abolished. From this we conclude that either the conformational change (step ℓ in Fig. 5) and/or the release of Na is electrogenic. If the Na-release is electrogenic one must assume an "ion-well" at the extracellular side of the pump.

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NON-EQUIVALENT CYTOPLASMIC Na^+ SITES AND THEIR SUSCEPTIBILITY TO TRANSMEMBRANE INTERACTION FROM EXTRACELLULAR Na^+.

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INTRODUCTION

The Na^+-Na^+ exchange accompanied by ATP hydrolysis is electrogenic with a stoichiometry of 3 cytoplasmic Na^+:2 extracellular Na^+:1 ATP suggesting that it proceeds via the same intermediate steps as Na^+-K^+ exchange, but with extracellular Na^+ acting at the normal K^+ sites (Lee and Blostein, 1980, Cornelius and Skou, 1985). The maximum rate of Na^+-Na^+ exchange is about 6% of Na^+-K^+ exchange.

Liposomes reconstituted with shark Na,K-ATPase engage in Na^+-Na^+ exchange accompanied by ATP hydrolysis in the absence of extracellular K^+. This preparation has been used in order to measure the cytoplasmic and extracellular Na^+-affinities, respectively and to investigate if these affinities are affected by the transmembrane Na^+-concentration. The effect of cations on the Na^+-Na^+ exchange is investigated by measuring ATP hydrolysis at saturating ATP concentrations as well as the Na^+ flux. The aim of the experiments has been to elucidate if the mechanism of cation activation and transport is ping-pong or simultaneous.

METHODS

The shark Na,K-ATPase is purified, solubilized in $C_{12}E_8$, and reconstituted into liposomes as previously described (Cornelius and Skou, 1984). The orientation of the enzyme in the proteoliposomes is 15% inside-out (i:o), 65% rightside-out (r:o) and the remainder 20% are incorporated

unsided (n:o) (Cornelius, 1987). The proteoliposomes used are 1:20 protein to lipid (wt:wt) and contain phospholipids and cholesterol in the following proportions (mole fractions) PC:PE:PS:chol (48:12:1.5:38.5).

The proteoliposomes contain 0.1 mM $MgCl_2$, 30 mM histidine (pH=7.0) and varying concentrations of NaCl (0 to 130 mM). The osmolarity is kept constant with sucrose. When ATP-hydrolysis is measured n:o-oriented enzyme molecules are inhibited by preincubation with Mg^{2+} (1mM), P_i (0.2 mM) and ouabain (1 mM). Using proteoliposomes of different Na^+-composition the activation (v) of Na^+-Na^+ exchange on i:o oriented enzyme molecules measured as rate of ATP hydrolysis or influx of ^{22}Na (cellular efflux) by cytoplasmic Na^+ is analysed according to the sequential interaction model of Adair-Pauling (Segal, 1975) in which each step in the binding sequence of a ligand (X) to enzyme (E) is characterized by a seperate intrinsic association konstant K'_i:

$$(1) \quad nY = \frac{\sum_{i=1}^{n} i(EX)_i}{\sum_{i=0}^{n} (EX)_i}$$

which for a 3-site (n=3) model conforms to:

$$(2) \quad Y = \frac{K'_1X + 2K'_1K'_2X^2 + K'_1K'_2K'_3X^3}{1 + 3K'_1X + 3K'_1K'_2X^2 + K'_1K'_2K'_3X^3}$$

The fractional saturation of the enzyme (Y) is estimated as v/V_{max}. The intrinsic association konstant K'_i is related to the thermodynamic constant K_i by a statistical factor:

$$(3) \quad K_i = \left(\frac{n + 1 - i}{i}\right) K'_i$$

RESULTS AND DISCUSSION

By varying the cytoplasmic Na^+-concentration at different fixed extracellular Na^+-concentrations in the absence of K^+ a series of activation curves are obtained which are all clearly sigmoid. Fig. 1 shows activation curves with either 2 mM or 130 mM extracellular Na^+ . The hydrolysis of ATP is exclusively due to i:o-oriented enzyme molecules since r:o-oriented enzyme is devoid of ATP and n:o-oriented enzyme is inhibited from the preincubation (see METHODS). The curves shown represent the best computerfit to the experimental data (*) using the Adair-equation (eq. 1) with 3 cytoplasmic Na^+-sites (eq. 2).

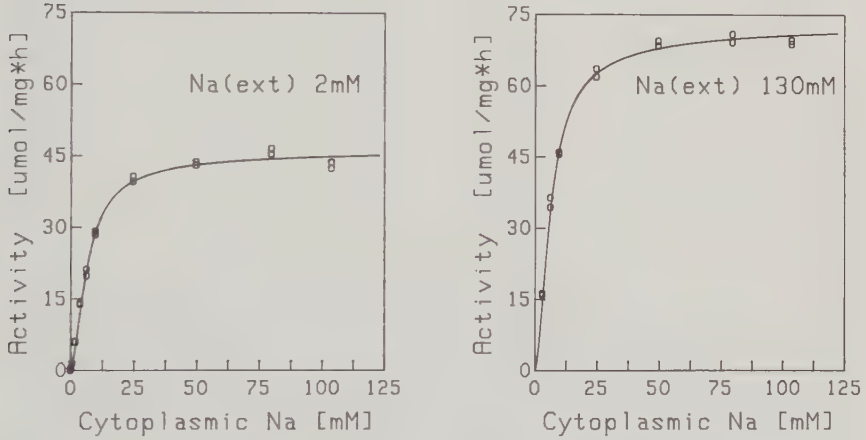

Fig. 1. Activation of ATP-dependent Na^+-Na^+ exchange by cytoplasmic Na^+ at two fixed extracellular Na^+-concentrations: 2mM and 130 mM.

All activation curves with fixed extracellular Na^+ (0 to 130 mM) are sigmoidal and fit the 3-site model. The fitting parameters for the different fixed extracellular Na^+-concentrations are collected in Table I below.

As indicated the activation by cytoplasmic Na^+ demonstrate positive cooperative interaction at all fixed extracellular Na^+-concentrations tested. E.g. at 130 mM extracellular Na^+ the intrinsic site dissociation constants are 40.2 mM, 3.5 mM , and 3 mM; values not very different from the values estimated by Foster and Ahmed (1976).

Table I. The fitting parameters computed from the Adair eq. with 3 cytoplasmic Na^+-sites at different fixed extracellular Na^+-concentrations. The figures are mean from 4 experiments with 1 S.E. indicated.

Na^+-conc. mM	K'_1	K'_2 mM	K'_3	$K_{0.5}$ mM	V_{max} µmol/mg·h
2	26.9±0.8	3.9±0.3	4.0±0.2	6.9±0.14	45.7±1.4
5	23.2±1.7	4.4±0.8	4.3±0.8	6.8±0.03	64.0±1.8
10	13.5±1.4	7.5±0.6	2.4±0.2	6.4±0.09	61.6±2.4
25	14.6±0.9	9.9±1.0	1.6±0.3	6.4±0.09	66.1±1.5
50	15.2±2.1	7.0±1.5	3.5±0.6	6.7±0.12	72.2±1.7
80	24.8±3.2	4.7±1.3	3.6±0.5	6.2±0.11	86.1±3.6
130	40.2±2.5	3.5±0.9	3.0±0.5	6.7±0.11	73.6±1.3

The observation that the activation curves for cytoplasmic Na^+ at different fixed extracellular Na^+ can be accounted for by assigning different values for the intrinsic site dissociation constants indicate that extracellular Na^+ has an allosteric effect on the binding constants of cytoplasmic Na^+, i.e a simultaneous binding of cytoplasmic and extracellular Na^+. However, it cannot be decided if the extracellular Na^+ is bound at transporting sites, which normally transport K^+, or to some regulatory sites that are not transport sites, or both. Binding of extracellular Na^+ to allosteric sites that are not transport sites have previously been shown by Karlish and Stein (1985).

Further support for a simultaneous binding of cytoplasmic and extracellular Na^+ is given by the observation that V_{max} increases with an increase in the extracellular Na^+-concentration whereas $K_{0.5}$, the apparent dissociation constant for half-maximal activation, is constant about 6.6 mM, Table I. According to the analysis of bisubstrate reactions by Cleland (1963) such kinetic pattern suggests that extracellular and cytoplasmic Na^+ are bound simultaneously in some part of the reaction cycle. The

kinetic pattern agrees with that obtained by Hoffman and Tosteson (1976) measuring Na^+-K^+ exchange and so does the conclusion. It is however in disagreement with the conclusion reached by Sachs (1986) that the reaction is ping-pong.

Using the same data as used for Table I the activation by extracellular Na^+ at different fixed cytoplasmic Na^+ can be analyzed by replotting the data assigning extracellular Na^+ as the independent variable. The curves are less reliable, however, since each point now corresponds to an individual liposome batch. The results with 10 mM cytoplasmic Na^+ is depicted in fig. 2A. The activation curves are qualitatively identical at all fixed cytoplasmic Na^+-concentrations tested.

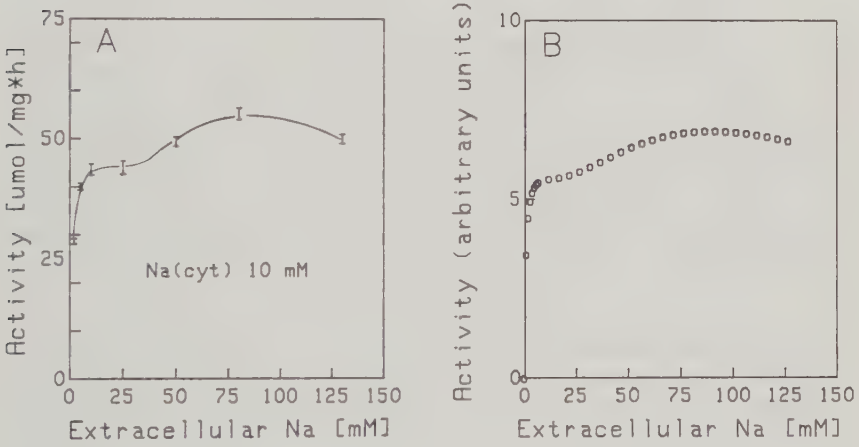

Fig. 2. A, The activation of ATP-dependent Na^+-Na^+ exchange by extracellular Na^+ at a fixed cytoplasmic Na^+-concentration of 10 mM. Bars indicate 1 S.E.
B, Computersimulated activation curve using the model depicted in fig. 3. The constants are given in the text.

As indicated in fig. 2A the activation pattern is complicated: At low extracellular Na^+ the activation is steeply increasing until a plateau is reached between 5-10 mM Na^+. By further increase in extracellular Na^+ the activity increases, passes through a maximum and then decreases for concentrations of extracellular Na^+ above 80 mM.

Plausible explanations for activation curves which exhibit characteristic bumps have been given by Levitzki and Koshland (1969): They emphasize that if the enzyme shows negative cooperative with regard to ligand binding at the same time as positive cooperativity in the rate of catalysis a distinct leveling occurs in the saturation plot.

The activation curves for extracellular Na^+ is analyzed using the rationale of Levitzki and Koshland. The model is shown in fig. 3 below: k_1 is 300 s^{-1}, k_{-1} is 5 s^{-1}; dephosphorylation is initiated by high-affinity (K_{Na1}=1 mM) binding of Na^+ to E_2P; E_2PNa dephosphorylates with a rate constant, k_2, of 7.5 s^{-1}. E_2PNa binds Na^+ to an allosteric site with a low affinity, K_{Na2} is 60 mM, but this form is unable to dephosphorylate; this is in agreement with previous suggestions of inhibitory extracellular Na^+ sites (for references see Kaplan (1982)). Binding of a third Na^+ with a low affinity, K_{Na3} is 80 mM, to a second transport site initiates dephosphorylation with a rate constant, k_3, of 22 s^{-1}. The dephosphorylation leads to occlusion, transfer to the membrane phase, and the following deocclusion to the cytoplasmic side of one respective two Na^+ per Pi released. There is a spontaneous dephosphorylation of E_2-P, an uncoupled Na^+ efflux, but this is only a small fraction of the Na^+-Na^+ exchange, about 10%.

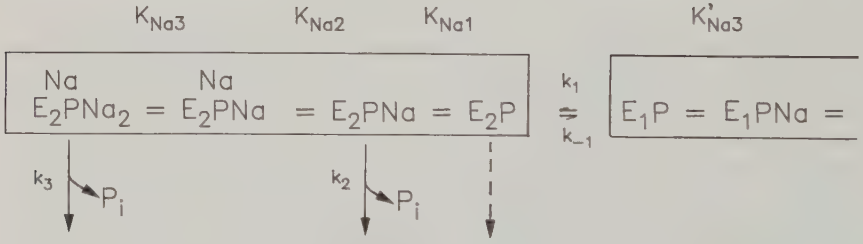

Fig. 3. Model for extracellular Na^+ activation. The enzyme species enclosed in boxes are assumed to be in rapid equilibrium. K_{Na} and k indicate dissociation- and rate-constants, respectively.

The deactivation seen at high extracellular Na^+ is accounted for in the model by stimulation of the backward reaction with a K'_{Na3} of 25 mM. A computer simulated activation curve using this model is shown in fig. 2B and compares rather well with the experimental curve in fig. 2A.

If the explanation of the extracellular Na^+ activation is correct it means that the stoichiometry is not strictly 3:2:1 (Na_{cyt}:Na_{ext}:ATP), but that the stoichiometry and the rate of dephosphorylation is dependent on the Na^+-concentration. This agrees with the observation of Yoda and Yoda (1987) that there are two phosphorylation-dephosphorylation cycles. One which transports one Na^+ from extracellular to intracellullar and another which transports two Na^+. It also agrees with the observation by Beauge and Glynn (1979) that the rate of dephosphorylation of enzyme prephosphorylated in the presence of Na^+ depends on the concentration of Na^+ during the dephosphorylation. With an intermediary concentration of Na^+, 5 mM, the rate of dephosphorylation is lower than with a higher as well as with a lower concentration of Na^+.

CONCLUSION

3 Na^+ cooperate positively for activation on the cytoplasmic side of the Na,K-ATPase. In the ATP hydrolysis dependent Na^+-Na^+ exchange the cytoplasmic intrinsic site constants depend on the concentration of extracellular Na^+, indicating an allosteric effect of extracellular Na^+ and that there is a simultaneous binding af cytoplasmic and extracellular Na^+. This find further support in the observation that $K_{0.5}$ for cytoplasmic Na^+ for activation is independent of extracellular Na^+, while V_{max} increases with extracellular Na^+. Extracellular Na^+ activates along a biphasic curve which can be explained from a model in which E_2-P binds Na^+ with a high affinity but has a low rate of dephosphorylation. Binding af Na^+ with a low affinity to an allosteric site on E_2-PNa leads to inactivation of dephosphorylation, while binding af a third Na^+ with low affinity to a second transport site gives a higher rate of dephosphorylation than of E_2-PNa.

ACKNOWLEDGEMENTS

Birte Westergård and Hanne Zakarias are gratefully acknowledged for technical assistance. This investigation was supported from The Danish Medical Research Council.

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THE Na/K PUMP OF LK SHEEP ERYTHROCYTES: MODULATION BY A MEMBRANE ANTIGEN

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INTRODUCTION

Erythrocytes from sheep of the LK phenotype (low [K], high [Na] cells) have a membrane antigen, L, which interacts with the Na/K pumps of these cells. The evidence is that antibodies against these antigens cause a several-fold stimulation of the pump (Ellory & Tucker, 1969). An interpretation of this observation is that the antigen is an inhibitor of the pump, and binding of anti-L antibody relieves the inhibition. In this report the basis of these interactions is described. From studies on the kinetics of the pump in intact cells before and after treatment with antibody, it was found that in untreated cells, K_c is a strong noncompetitive inhibitor of the pump. The antibody abolishes this inhibition, thereby stimulating the pump. This effect is specific in that no other kinetic parameters of the pump were affected. At the same time that anti-L modifies K_c binding, anti-L binding is modified by K_c. Full reports of some of these results appear elsewhere (Farquharson & Dunham, 1986; Dunham & Anderson, 1987).

MATERIALS AND METHODS

Cells were obtained from LK Suffolk sheep. Cellular cation concentrations were altered using the p-chloromercuribenzene sulfonate (PCMBS)-dithiothreitol method.

$[Na]_c$ and $[K]_c$ were varied independently using choline as an inert substitute. In a few experiments the nystatin method was used to vary $[K]_c$. Unidirectional K influxes were measured using ^{86}Rb. The medium contained (mM): NaCl (145), KCl (5), Tris-HCl (10), pH 7.5. The pump flux was defined using 0.1 mM ouabain.

Numbers of pumps per cell were obtained from simultaneous measurements of ^{3}H-ouabain binding and extent of inhibition of the pump. Exposure times to ouabain (0.3 uM) were varied to give three levels of inhibition, 30 to 80%. Ouabain molecules bound per cell plotted against percent inhibition gave linear correlations. Extrapolation to 100% inhibition gave number of functioning pumps per cell.

Kinetics of pump fluxes were studied by varying $[Na]_c$. Sigmoid curves were fitted to the Hill equation, or to a form of the Hill equation containing terms for competitive and noncompetitive inhibition (Albers and Koval, 1973). Curves were fitted to the data using iterative computer programs for non-linear least squares fits. For complete methods see Dunham and Anderson (1987).

RESULTS

Treatment with anti-L increases the number of functioning pumps about 2-fold, as shown in Table 1, the results of nine paired experiments. This differs from the results of Joiner & Lauf (1978); however in this latter study number of ouabain binding sites may have exceeded number of functioning pumps. Furthermore Joiner & Lauf (1978) present some evidence, not commented upon, that anti-L can increase the number of pumps.

Table 1. Numbers of functioning Na/K pumps per LK sheep red cell at physiological $[K]_c$ measured using ^{3}H-ouabain before (control) and after treatment with anti-L antibody.

cells	($[K]_c$)	pumps/cell control	anti-L
fresh	(12 mmol/l)	41 ± 8 (9)	85 ± 11 (9)
PCMBS	(10 mmol/l)	53 ± 8 (7)	85 ± 12 (7)

The effect of anti-L on numbers of pumps was studied further in cells with altered cation concentrations. Table 1 shows that PCMBS treatment itself doesn't alter numbers of pumps.

Figure 1 shows that lowering $[K]_c$ raises the number of functioning pumps in control cells, but not in anti-L-treated cells. Furthermore at near zero $[K]_c$ anti-L does not affect numbers of functioning pumps.

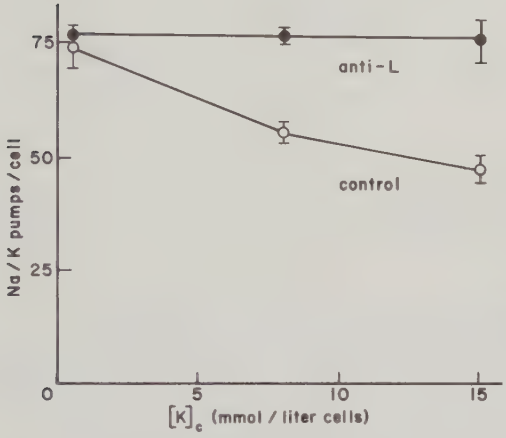

Figure 1. Effect of lowering $[K]_c$ on numbers of Na/K pumps per cell, control and after anti-L treatment. Reproduced from the Journal of General Physiology, 1987, Vol. 90, pp. 3-25.

Three predictions were made from this result: 1) K_c is a noncompetitive inhibitor of the pump in LK cells; 2) anti-L relieves this noncompetitive inhibition; 3) K_c modifies anti-L binding.

To test the first prediction, pump kinetics were measured in control cells. Figure 2 shows the results of 10 experiments in which pump K influx was measured in cells with $[Na]_c$ (substrate) varied at three fixed levels of $[K]_c$ (inhibitor).

The results suggest three conclusions. First, the curves are sigmoid, as expected (Na binds at three loading sites per pump), and K_c is inhibitory. The surprising result is that the apparent maximum velocity (J'_{max}) was reduced by K_c (statistically significant, $P<0.05$, from asymptotic SEs). This demonstrates noncompetitive inhibition and confirms prediction 1) made from the results in Figure 1.

Further analysis of the results in Figure 2, made by a replot of J'_{max} vs $[K]_c$ and by using the Hill-Albers equation, permitted calculation of the following constants:

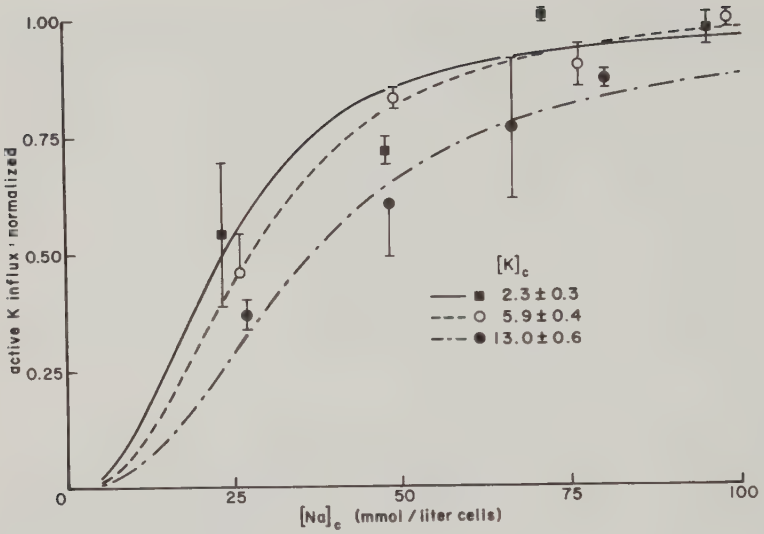

Figure 2. Kinetics of activation of the Na/K pump in control cells. Results were normalized by setting at 1.0 the flux in each experiment at the highest $[Na]_c$, and lowest $[K]_c$. The curves are computer fits to the means obtained using the Hill equation. Reproduced from the Journal of General Physiology, 1987, Vol. 90, pp.3-25.

the Hill coefficient, 2.5; the Michaelis constant for Na_c, 28.6 mM; the constant for K_c as a noncompetitive inhibitor 3.4 mM, and the constant for K_c as a competitive inhibitor, 8.0 mM. These results confirm the demonstration of Glynn & Ellory (1972) that in LK cells K_c has a higher affinity as an inhibitor of the pump than Na_c has as a substrate; Glynn & Ellory did not realize that K_c is a noncompetitive as well as a competitive inhibitor.

To test the second prediction, kinetics of the pump were measured in anti-L-treated cells. The results shown in Figure 3 are from four experiments carried out the same way as those in Figure 2.

As with control cells, the curves are sigmoid, and K_c inhibits. However the J'_{max}'s were all the same (within 6%), indicating that K_c is not a noncompetitive inhibitor. This confirms prediction 2) above, that anti-L abolishes noncompetitive inhibition by K_c.

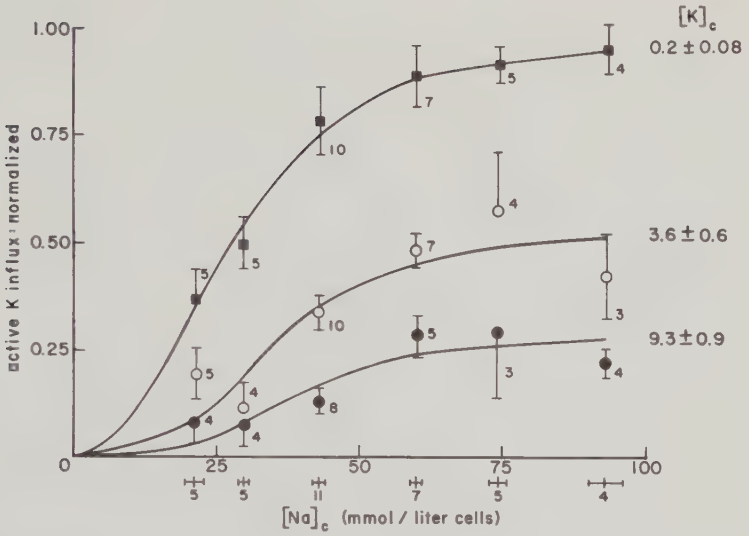

Figure 3. Kinetics of activation of the Na/K pump in anti-L-treated cells. Normalization and curve fitting were the same as in the experiments in Figure 2. Reproduced from the Journal of General Physiology, 1987, Vol. 90, pp.3-25.

Kinetic constants were calculated by computer fits to the data in Figure 3 using the Hill-Albers equation. These are presented in Table 2 along with the corresponding constants for control cells. Also shown are the true J_{max}'s for the two cell types and the apparent Michaelis constants for K_o as a substrate (from Dunham & Anderson, 1987).

Table 2. Kinetic constants for the Na/K pump of control and anti-L-treated LK sheep erythrocytes.

Cells	J_{max}	K_S^{Na}	K_S^K	K_{IN}	K_{IC}	n
control	0.63	29 ± 0.6	1.9	3.4	8.0 ± 1.0	2.5 ± 0.1
anti-L	1.00	23 ± 2.1	1.7	00	9.3 ± 1.4	(2.5)

J_{max}: true maximum pump flux (normalized); K_S^{Na} and K_S^K: Michaelis constants for Na_c and K_o; K_{IN}: constant for K_c as noncompetitive inhibitor; n: Hill coefficient (assumed for anti-L cells). Units (apart from J_{max} and n): mM. Errors: asymptotic SEs.

The results in Table 3 show that the effect of anti-L on the pump is highly specific. Noncompetitive inhibition by K_c is abolished, but no other affinity is affected. (This justifies the assumption that K_c inhibits in control cells at two distinct sites.)

The effect of anti-L on K_{IN} explains much of the stimulation of the pump at physiological $[K]_c$, at which stimulation can exceed 4-fold (Ellory & Tucker, 1969; Dunham & Anderson, 1987). However anti-L raises true J_{max} about 60%, obviously not a consequence of an effect on K_{IN}. This stimulation of J_{max} seems not to be an effect on affinities for Mg, Ca, or ATP (Dunham & Anderson, 1987; Lauf, this volume). It may reflect an increase in intrinsic turnover number of the pump.

The third prediction from Figure 1 was that, if anti-L modifies K_c affinity of the pump, then varying $[K]_c$ should modify anti-L binding. Anti-L binding could not be measured directly since the number of L antigens per cell exceeds the number of pumps by more that an order of magnitude (Smalley et al., 1982). Therefore anti-L binding was measured indirectly by its effect on the pump flux. The general strategy was to make cells with either high or zero $[K]_c$, expose them to anti-L, then make all the cells zero $[K]_c$ prior to measuring the pump flux (the pump could not be measured in the high $[K]_c$ cells). Because it acts in minutes rather than days, nystatin rather than PCMBS was used to vary $[K]_c$. The details of the experimental design and the results are shown in Figure 4.

The "control" pump fluxes were the same in cells which had been 63 mmol/l $[K]_c$ and in 0 $[K]_c$ cells. Anti-L stimulated the pump in both, but to a considerably greater extent, 60% (45 umol/lxh), in cells which had 63 mmol/l $[K]_c$ during exposure to anti-L than in cells which had zero $[K]_c$. This was shown to be an effect on extent of anti-L binding (Farquharson & Dunham, 1986); rate may have been affected as well.

Thus anti-L, binding to antigens at the external cell surface, reduces the binding of K_c intracellularly to pumps adjacent to the antigens. At the same time, K_c promotes binding of the antibody. This interaction can be understood in terms of a reversible membrane complex between pump and antigen, L-P, to which both K_c and the antibody, A, bind preferentially. However the antibody promotes dissociation of the complex, thereby reducing K_c binding, whereas K_c stabilizes the complex, and this promotes antibody binding.

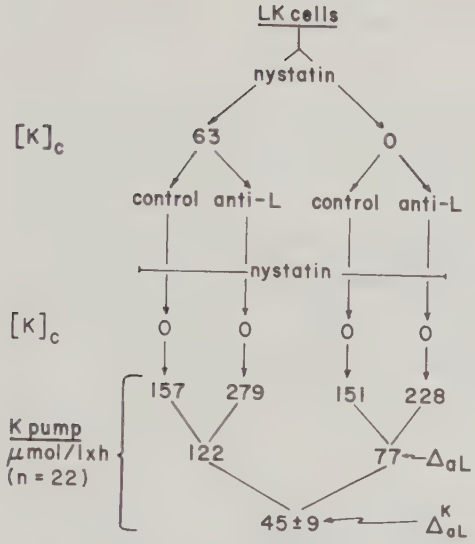

Figure 4. Effect of $[K]_c$ on anti-L binding to LK cells: protocal and results. Δ_{aL}: effect of anti-L on pump flux; Δ^{K}_{aL}: effect of high $[K]_c$ on anti-L-stimulated pump flux.

This scheme is summarized as folows:

$$A + L\text{-}P\text{-}K \text{ -----> } A\text{-}L + P + K$$

In this way the first ligand, A (anti-L), can reduce the binding of the second, K_c, but the second ligand promotes the binding of the first.

CONCLUSIONS

In LK sheep red cells at physiological $[K]_c$, anti-L antibody increases the number of functioning pumps about 2-fold. Reducing $[K]_c$ also increases this number in control cells, but not in anti-L-treated cells. From these results it was predicted, and then demonstrated, that K_c is a noncompetitive inhibitor of the pump. This highly unusual interaction of K_c with the pump may be entirely a consequence of the inhibitory action exerted on the pump by the L antigen; alternatively a subunit of the pump of LK cells may be an unusual isoform.

It was also predicted that the stimulation of the pump by anti-L was due in large part to the relief of noncompetitive inhibition by K_c. This prediction was also confirmed. Indeed the alteration in kinetic properties of the pump was highly specific in that the affinity of the Na

loading site was not altered either for the substrate or for K as an inhibitor.

Finally it was predicted that K_c modulates anti-L binding; this too was confirmed, and with the surprising result that, while anti-L reduces K_c binding, K_c promotes anti-L binding. An explanation was offered in terms of an interaction of pump and antigen in a dissociable complex in the membrane. K_c stabilizes the complex, and in so doing promotes the inhibited configuration. Anti-L destabilizes the complex, thereby reducing K_c binding and stimulating the pump. This interaction between membrane receptor (antigen), pump, and extra- and intracellular ligands may prove a useful model for signal transduction systems.

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PUMP CURRENTS GENERATED BY RENAL NA^+K^+-ATPASE AND GASTRIC H^+K^+-ATPASE ON BLACK LIPID MEMBRANES

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INTRODUCTION

The Na^+K^+-ATPase from mammalian cells is an electrogenic pump, which exchanges 3 Na^+ for 2 K^+ ions across the cell membrane. The electrical properties of the Na^+K^+-ATPase were investigated in whole cells (for a review see De Weer, 1986), in reconstituted vesicles (see e.g. Dixon and Hokin, 1980, Goldshlegger et al., 1987) and on black lipid membranes (BLM) (Fendler et al., 1985, Nagel et al., 1987, Borlinghaus et al., 1987). According to a Na^+/K^+ stoichiometry of 3/2 it is electrogenic and generates stationary pump currents.

The H^+K^+-ATPase occurs abundantly in the gastric mucosa (for a review see Sachs et al., 1978). Similar to the Na^+K^+-ATPase it can be described with the Albers Post scheme. The H^+K^+-ATPase exchanges H^+ against K^+ electroneutrally with a stoichiometry of 1/1 or 2/2. It is therefore expected to be electroneutral.

Adsorption of membrane fragments containing the ATPase to a BLM offers the possibility to monitor directly the charge translocation taking place during the ATPase pumping cycle. Pump currents were initiated by a light-induced concentration jump of ATP via the conversion of a photolabile inactive derivative, the caged ATP (Kaplan et al., 1978 and Forbush, 1985), into its active form. With this method the electrogenic transport activity of the Na^+K^+-ATPase could be detected with high sensitivity (0.5 pA) and

high time resolution (1 ms). Electrical events in the H^+K^+-ATPase were detected by separating the H^+ translocating steps from the K^+ transporting steps.

MATERIALS AND METHODS

Preparation of caged ATP and ATPase. The triethylammonium salt of caged ATP (P^3-1-(2-nitro)phenylethyladenosine 5'-triphosphate) was synthesized as reported previously (Fendler et al., 1985). Na^+K^+-ATPase was prepared from the red outer medulla of pig kidneys according to Jorgensen (1974). Membrane fragements in the form of cup shaped discs of 0.1 - 0.6 µm diameter were obtained. The suspension contained ca. 2.5 mg/ml protein with a typical specific activity of 30 µmol P_i mg^{-1} min^{-1} at 37° C. Membrane sheets enriched with H^+K^+-ATPase were prepared according the procedure of Ray and Forte, 1976., and Schrijen et al., 1980. The suspension contained ca. 3 mg/ml protein and had a specific activity of 1 to 2 µmol Pi mg^{-1} min^{-1}. Eventual activity of contaminating Na^+K^+-ATPase was suppressed by the addition of ouabain.

Lipid bilayer setup. Optically black lipid membranes with an area of 10^{-2} cm^2 were formed in a thermostated teflon cell with 1.5 ml of an appropriate electrolyte solution in each compartment. The temperature was kept at 22°C. The membrane forming solution contained 1.5 % w/v diphytanoyllecithin (Avanti Biochemicals, Birmingham Al. USA) and 0.025 % w/v octadecylamine (Riedel de Haen, Hannover, FRG) in n-decane. The membrane was connected to an external measuring circuit via platinum electrodes. To avoid photoeffects, the electrodes were separated from the aqueous compartments of the teflon cell by agar-agar salt bridges. The signals were filtered and recorded with a digital oscilloscope. For further details see Fendler et al., 1985.

15 µl of the Na^+K^+-ATPase or H^+K^+-ATPase containing suspension together with different amounts of caged ATP were added under stirring to one compartment of the cuvette, containing an appropriate electrolyte. To photolyse the caged ATP, light pulses of a Hg high pressure lamp of 125 ms duration or of an excimer laser (wavelength 308 nm) of 10 ns duration were focussed onto the membrane. One flash of the lamp or the laser converted 30 %

and 10 %, respectively, of the caged ATP in the membrane plane into ATP.

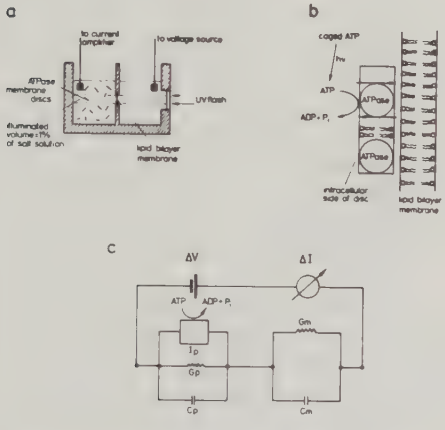

Figure 1: Schematic representation of the bilayer setup.
a: Teflon chamber with black lipid membrane (BLM) and adsorbed ATPase membrane fragments.
b: Proposed sandwich-like arrangement of discs and underlying lipid membrane. The two membranes are capacitively coupled.
c: Equivalent circuit diagram of the two membranes in series. G_m, G_p refer to the conductance of the planar bilayer (BLM) and the disc membrane, respectively, C_m and C_p to the capacitance of BLM and discs, respectively.

PUMP CURRENTS OF THE NA^+K^+-ATPASE

15 min after addition of ATPase and caged ATP a current could be induced by a UV light pulse. In the absence of ionophores the current is transient, whereas the permeabilization of the underlying lipid bilayer yields a sustained current (figure 2) which decays slowly to zero due to the consumption and the dilution of the liberated ATP into the not irradiated volume. Because of their insensitivity to UV light a carrier system consisting of monensin as an alkali metal ion/H^+ exchanger together with the protonophore 1799 was used for these experiments.

The sign of the current was always same and corresponds to a positive charge movement toward the protein free side of the BLM. The discs are preferentially oriented with the extracellular side facing the planar bilayer.

Mg^{++} was necessary for the appearance of pump currents. If not otherwise indicated, in all experiments a concentration of free Mg^{++} of 3 mM was used. Together with additional control experiments (Fendler et al., 1985) these experiments show that the observed current reflects

the pumping activity of the Na^+K^+-ATPase.

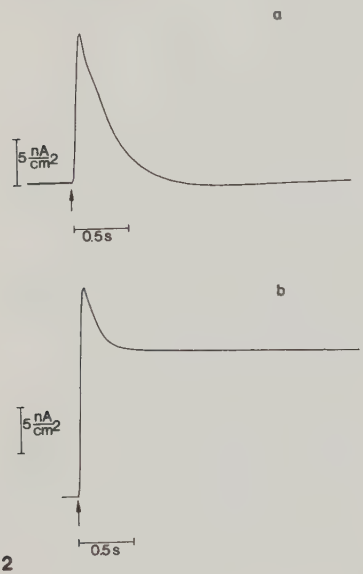

Figure 2: Short circuit currents in the absence and presence of ionophores. The membrane bathing solution contained 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 25 mM imidazole-HCL pH 7.5. The arrows indicate opening of the shutter, which was opened for 125 ms.
a: 100 µM caged ATP, no addition of ionophores.
b: Same membrane system after addition of monensin and 1799 to a concentration of 10 µM and 0.6 µM, respectively.

The appearance of stationary Na^+ pump currents can prove that the enzyme acts as an electrogenic Na^+ pump. They can be detected on the sandwich-like structure by increasing the conductivity of the supporting lipid bilayer with an appropriate carrier system. A stationary Na^+ pump current was obtained indeed after the addition of monensin and the protonophore 1799 to the membrane system (figure 4). It can be excluded that this effect is due to residual K^+ in the electrolyte (Nagel et al., 1987). The experiment therefore demonstrates the electrogenic activity of the Na^+-ATPase.

TRANSIENT KINETICS OF THE NA^+K^+ATPASE

Using a 10 ns light pulse of an excimer laser together with fast recording instrumentation the current generated by the Na^+K^+-ATPase after an ATP concentration jump could be measured with a time resolution of 1 ms. This provides at the same time information about the electrogenicity as well as the rate constants of partial reaction steps of the Na^+K^+-ATPase.

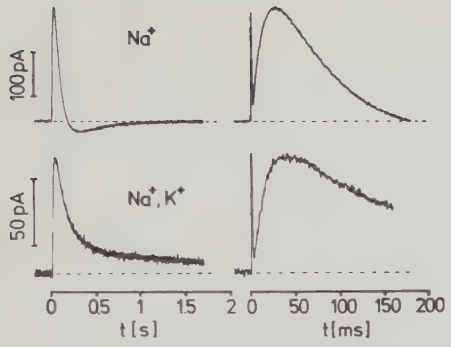

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Figure 3: Short circuit currents without and with K^+. The signal was recorded simultaneously with two different sample rates (left hand side and right hand side). Conditions: 3 mM $MgCl_2$, 25 mM imidazole-HCl at pH 6.3, 7 µM ATP released per flash, 130 mM NaCl and 20 mM KCl (lower traces).

Inspection of the equivalent circuit of the disc-BLM compound membrane (see figure 1) shows that the current $I_p(t)$ of the pump is distorted by the network formed by the disc-BLM compound membrane. In particular the characteristic time constant $\tau_o = (C_m+C_p)/(G_m+G_p)$ of the equivalent circuit is introduced in the measured signal (Fahr et al. 1981).

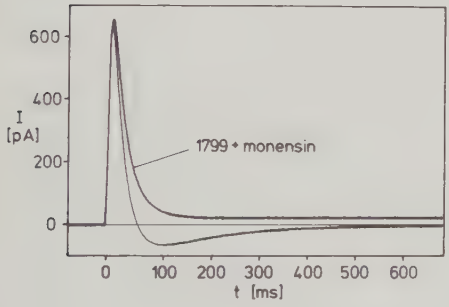

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Figure 4: Short circuit currents in the absence and presence of ionophores. Conditions as in fig.3 upper traces, i.e. without KCl.

With the data shown in figure 3 excellent fits with 3 exponential components were obtained. The slowest time constant τ_3 determined this way has to be assigned to the characteristic time constant τ_o of the equivalent circuit (Fendler et al., 1987). This is further supported by the experiment shown in figure 4. If the membrane conductivity is increased by the addition of ionophores, the slow negative component of the Na^+ current disappears.

The fast two time constants τ_1 and τ_2 reflect charge translocations during the reaction cycle of the enzyme. Because τ_3 is much larger than τ_1 and τ_2 distortion of the pump current by the network is small and will be neglected.

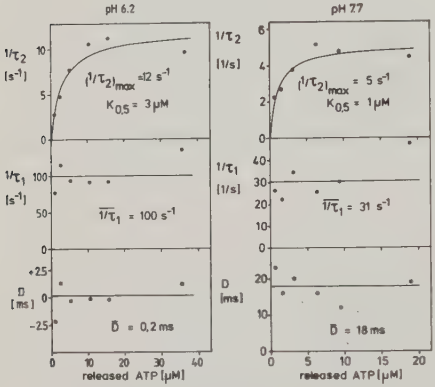


Figure 5: ATP dependence of the fit parameters of equation 1. The ATP dependence of the inverse time constants $1/\tau_1$ and $1/\tau_2$ and of the delay D (see equation 1) of the UV flash induced current was measured at pH 6.2 and pH 7.7. The electrolyte contained 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ different amounts of caged ATP and 25 mM imidazole at pH 6.2 and pH 7.7, respectively. For $1/\tau_2$ the solid line represents a fit of a Michaelis Menten type concentration dependence to the data. For $1/\tau_1$ and D the solid line is a horizontal line at the average value $1/\tau_1$ and D respectively.

The two intrinsic time constants of the Na^+K^+-ATPase found in the photoinduced current suggest a two step first order reaction sequence with an electrosilent transition followed by an electrogenic one. The current transient generated by this model is given by :

$$I(t) = \frac{A}{\tau_2-\tau_1} * \left(e^{-(t-D)/\tau_2} - e^{-(t-D)/\tau_1} \right)$$

The delay time D was introduced to account for the kinetics of ATP release from caged ATP. This function was fitted to the data at different ATP concentrations.

The saturating behaviour of $1/\tau_2$ shows that substrate binding is involved in this step and that it consequently has to precede the reaction characterized by τ_1. The situation is, however, complicated by the fact that caged ATP is a competitive inhibitor of the Na^+K^+-ATPase (Forbush, 1985 and Nagel et al., 1987).

The arguments given above support a kinetic model

consisting of a fast electrogenic step with rate constant 100 s^{-1} (pH 6.2) which is preceded by a non electrogenic slow process which takes place in approximately 80 ms and involves binding of the substrate. The similarity of the results in the absence and presence of K^+ suggests that the current is generated during the Na^+ dependent steps of the first half cycle:

$$E_1+ATP \rightarrow E_1 \cdot ATP \rightarrow E_1P \rightarrow E_2P$$

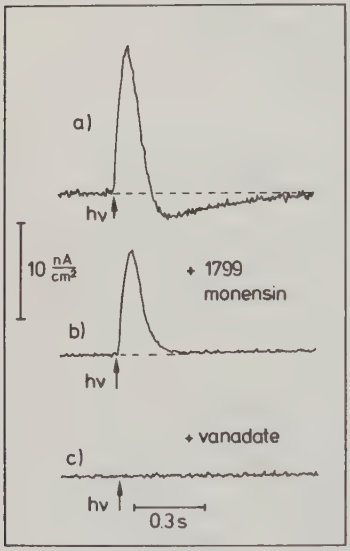
As already discussed τ_2 is attributed to substrate binding. τ_1 must then be due to reactions of the phosphoenzymes. Assignment of the observed transition by comparison with the rate constants reported in the literature is not possible because of differences in enzyme provenience and electrolyte composition. The decision which of the phosphoenzyme transitions is described by τ_1 has therefore to remain open.

ELECTROGENIC EVENTS DURING THE H^+K^+-ATPASE CYCLE

Under physiological electrolyte conditions (20 mM K^+ 120 mM Na^+, 3 mM Mg^{2+} pH 7.0) the adsorbed membrane sheets with H^+K^+-ATPase did not show any electrical response after a light induced ATP concentration jump (10 µM). If, however, K^+ and Na^+ were removed, a transient ATP dependent current was observed. The addition of the protonophores did not induce a stationary current (fig. 6b). Vanadate inhibited the signal completely (fig. 6c). The sign of the transient current was the same in all experiments and corresponded to a movement of a positive charge towards the protein free side of the planar lipid membrane. The oppositely directed relaxation of the current (fig. 6a) is related to the off response of the capacitively coupled system similarly as described above for the Na^+K^+-ATPase. Consequently after the addition of the ionophores the discharging effect disappeared, because of the high conductivity of the BLM under those conditions.

The reduction of the transient currents after addition of K^+ is shown in figure 6d. Since at high K^+ the enzyme occurs predominantly in the E_2K form, activation of the phosphorylation starting from the E_1 form is reduced. The electrical signal can be restored by the addition of higher amounts of ATP (figure 6d). A similar behaviour has been

reported for the Na^+K^+-ATPase. At physiological K^+ concentrations (20 mM) no electrical response was observed even at an ATP concentration of 0.3 mM. This implies that the $E_2K \rightarrow E_1$ step is slow compared to the phosphorylation reaction.



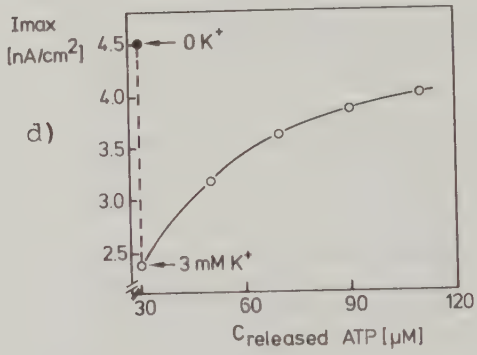


Figure 6: Electrical response of H^+K^+-ATPase. Membrane bathing solution contains 3 mM $MgCl_2$ and 50 mM imidazole pH 6.0. The arrows indicate the opening of the shutter.
a: 100 μM caged ATP. b: Same membrane system after addition of 1 μM 1799 and 10 μM monensin. c: 100 μM caged ATP and o.2 mM vanadate. d: K^+/ATP antagonism.

CONCLUSIONS

Detailed information about charge transport in ion translocating membrane proteins may be obtained with the method presented in this article. For both the Na^+K^+-ATPase and the H^+K^+-ATPase the peak currents observed in the disk-BLM system reflect early events in the reaction cycle, probably the $E_1 \rightarrow E_2P$ half-cycle. This is illustrated for the Na^+K^+-ATPase by the arrow in figure 7.

In addition, stationary currents give complementary information about slow steps of the cycle and the overall electrogenic activity of the enzyme. These experiments have been used to show that the Na^+K^+-ATPase functions as an

electrogenic pump in the presence and absence of K^+ and that the H^+K^+-ATPase is not electrogenic.

From fast current measurements it is concluded, that the electrogenic step observed for the Na^+K^+-ATPase (rate constant 100 s^{-1}) is one of the phosphoenzyme transitions: E_1 -> E_1P -> E_2P. The preceding nonelectrogenic slow step (80 ms) probably reflects ATP binding and exchange with caged ATP. No evidence has been found for a K^+ dependent electrogenic transport of ions back to the cytoplasmic side.

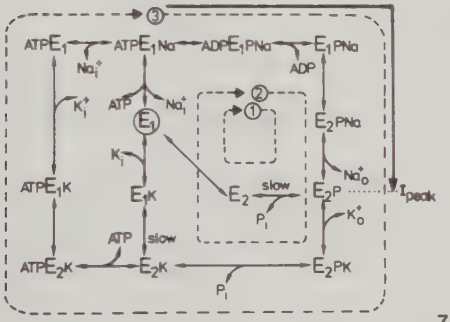


Figure 7: Reaction cycle of the Na^+K^+-ATPase (modified scheme from Karlish et al., 1978). Loop 1 shows the Na^+-ATPase cycle; Loop 2 the Na^+K^+-ATPase cycle at low ATP concentrations; loop 3 the physiological case, where the low affinity ATP binding site is saturated.

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Comparing the Na^+K^+-ATPase and H^+K^+-ATPase a very similar behaviour is found. However, the dephosphorylation and concomitant K^+ transport in the H^+K^+-ATPase apparently is different with respect to electrogeneicity. As shown recently by Goldshlegger et al. (1987), the K^+ transport in Na^+K^+-ATPase is electroneutral. Since the H^+ transport in the H^+K^+-ATPase is electrogenic (see above), the K^+ transport has to be electrogenic too, because of the overall electroneutral behaviour of the enzmye under stationary conditions.

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EFFECTS OF CA IN THE PRESENCE AND ABSENCE OF MG ON THE CYTOPLASMIC ASPECT OF THE NA/K PUMP IN HIGH K SHEEP RED CELLS

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INTRODUCTION

Much of the work to elucidate the nature of the genetic cation dimorphism in sheep red cells has focused on monovalent cations and their regulation through active and passive transport to maintain cellular volume (Tosteson and Hoffman 1960, Hoffman and Tosteson 1971, Dunham and Hoffman 1971, Joiner and Lauf 1978a,b, for review see Lauf 1982). Thus high K (HK) red cells differ from low K (LK) red cells by virtue of pump kinetics and higher pump numbers, and by lower ouabain-resistant K fluxes. Of particular interest are the kinetic differences of the Na/K pump between HK and LK red cells which arise by an unknown mechanism during maturation to the final erythrocyte (Lee, Woo and Tosteson 1966, Dunham and Blostein 1976, Lauf et al. 1978). It was suggested that there are differences in the affinities for ATP in HK and LK pumps (Blostein and Whittington 1973). Alternatively one may postulate that bivalent cations known to be required for the ATP dependent ion translocation may affect differently the pump molecules of HK and LK cells.

We recently studied the effect of varying cellular Mg ions, Mg_c, on the Na/K pump and found that HK and LK red cells, the latter also in the presence of anti-L_p (Ellory and Tucker, 1969, Lauf et al. 1970) did not differ in their response to alteration of Mg_c (Fujise and Lauf, 1986, and manuscript submitted elsewhere). In the context of these studies we found that elevating cellular Ca, Ca_c, inhibited 50% of the Na/K pump in both HK and LK sheep red

cells treated with A23187 to adjust the concentrations of Ca_c, $[Ca]_c$, and Mg_c, $[Mg]_c$, at about 0.7 mmol $[Ca]_c$/liter cells and normal $[Mg]_c$ (usually around 1.8 mmol/liter cells).

Here we present further data on the inhibitory action of Ca_c elevated by A23187 in presence or absence of Mg_c in HK red cells. Our findings of an uncompetitive inhibition by Ca_c of ouabain-sensitive Rb influx suggests an effect of Ca at the E_2K form of the pump cycle.

MATERIAL AND METHODS

Ouabain sensitive Na/K pump fluxes in HK sheep red cells were measured with Rb as K congener, in the absence of Na with glucamine as replacement cation to exclude Na effects at the K loading site of the pump (Hoffman and Tosteson, 1971), and with NO_3 replacing Cl to reduce volume-sensitive K:Cl cotransport (Fujise and Lauf 1987). Intracellular Ca and Mg activities were varied with A23187 ensuring equilibrium levels following previous work on human red cells by Ferreira and Lew (1976) and Brown and Lew (1983). It should be pointed out that cellular ATP levels remained little changed since sheep red cells are endowed with only a very small Ca-pump (Eaton et al. 1978). Quantitative determinations of cellular Rb, Ca and Mg was done by atomic absorption spectrophotometry using a Perkin Elmer 5000. Procedural details are being published elsewhere (Fujise and Lauf, manuscript submitted elsewhere).

RESULTS AND DISCUSSION

Figure 1 shows $^{i}M^{P}_{Rb}$, the Na/K pump mediated Rb influx as function of increasing medium Ca concentrations, $[Ca]_o$, at constant external Mg concentration, $[Mg]_o$, of 10^{-4}M in A23187 treated HK red cells. At $[Ca]_o > 10^{-5}$M, inhibition of $^{i}M^{P}_{Rb}$ occurred with 15% pump activity left at 10^{-3}M $[Ca]_o$, independent of $[Rb]_o$ used in the flux experiment. Figure 2 shows that the inhibiting effect of 5 x 10^{-5}M $[Ca]_o$ can be overcome by increasing $[Mg]_o$ to 10^{-3}M. These data suggest that Ca has entered the cell through A23187 and inhibited the Na/K pump, an effect which is reversible by raising Mg_c via the A23187 method. The inhibitory effect was Ca_c specific and not due to Mg_c depletion since even at 5 x 10^{-4}M $[Ca]_o$ there was a reduction of $[Mg]_c$ by only 20%. In experiments involving

HK and LK sheep red cells we found that with about 10^{-4}M $[Mg]_o$ the Ca level yielding 50% inhibition of the $^iM^P_{Rb}$ was 0.7 mmol/liter celis (Fujise and Lauf, manuscript submitted, elsewhere).

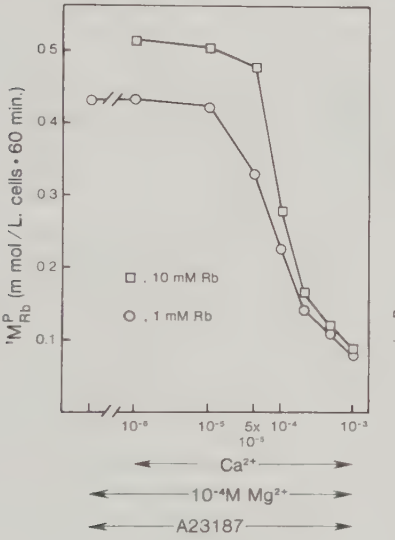

Figure 1

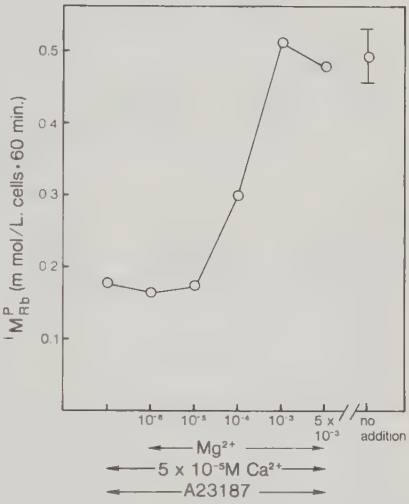

Figure 2

Fig. 1 Effect of various concentration of Ca_o at constant medium $[Mg]_o$ in presence of 8 µM A23187 on K^+ pump Rb influx, $^iM^P_{Rb}$, in HK sheep red cells at high and low external Rb concentrations.

Fig. 2 Effect of various concentration of Mg_o at constant medium $[Ca]_o$ ($5x10^{-5}$M) in presence of 8 µM A233187 on K^+ pump Rb influx, $^iM^P_{Rb}$, in HK sheep red cells at 10 mM $[Rb]_o$.

The mechanism by which Ca_c inhibits the Na/K pump was addressed in the kinetic experiment shown in Figure 3 which in A) plots $^iM^P_{Rb}$ as functions of $[Rb]_o$ and in B) displays the data of A) as a Hanes Plot. Note the dramatic reduction of v and the left shift of the Rb activation curve in A) which translates in B) into both a V_m change (slope = $1/V_m$) and a decrease in K_m (x-intercept), hence represents an uncompetitive inhibition. Table 1 shows the effects of Ca_c on K_m and V_m in A23187 treated HK cells in absence and presence of Mg as compared to untreated controls.

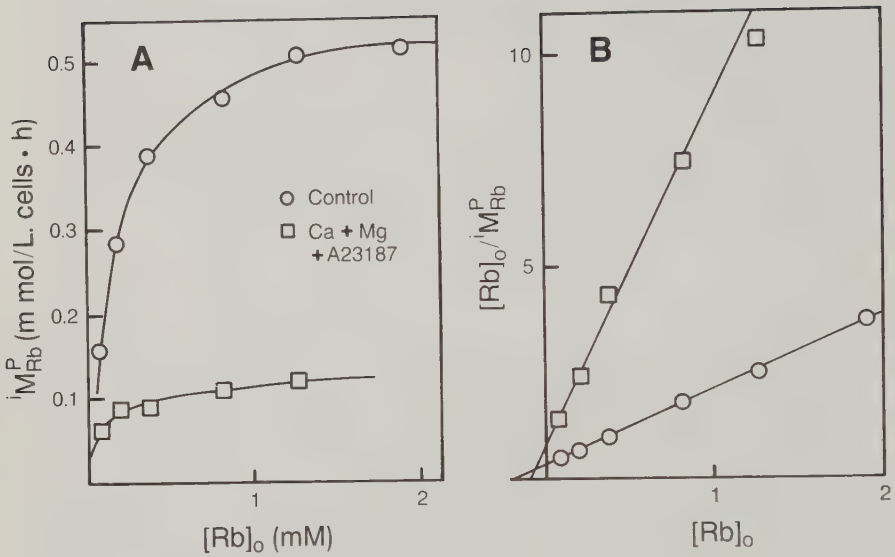

Fig. 3 A. Effect of intracellular Ca at constant Mg on Rb^+ pump influx, $^{i}M^{P}_{Rb}$, as function of medium Rb^+ concentration, $[Rb]_o$, in HK sheep red cells. The cells were washed after treatment with 2×10^{-4}M Ca, 1.1×10^{-4}M Mg and 8 µM A23187, then incubated in flux medium without Ca, Mg and A23187. B. A Hanes plot of values from the experiment in A. Lines calculated by regression analysis.

Table 1

Effect of Ca with or without Mg in presence of A23187 on kinetic parameters in HK sheep red cells.

Parameter	no addition	5×10^{-5} M Ca 8 µM A23187	2×10^{-4} M Ca 1.1×10^{-3}M Mg 8 µM A23187
	(n=3)[a]	(n=2)[b]	(n=2)[b]
Km (mM)	0.218±0.034	0.081±0.010	0.101±0.020
V_{max} (mmol/L cells"h)	0.510±0.022	0.134±0.005	0.116±0.002

a) Means and SD. b) Means and ranges.

Although the active pump fluxes are small, the sheep erythrocyte offers a suitable model to study with the aid of A23187 the effect of intracellular Mg and Ca changes because sheep red cells lack for example the Ca-induced K channel (Brown et al. 1978), possess only a small Ca pump (Eaton et al. 1978) and hence ATP remains constant throughout A23187 treatment (Lauf, 1985), and most of the ouabain-resistant K fluxes are inhibitable by replacing Cl with NO_3 (Lauf, 1982). Naturally, for these reasons studies dealing with changes in Ca_c in intact human red cells are more complex and hence less numerous (Brown and Lew 1983; Flatman and Lew 1981; Gardos et al. 1977).

In the present study we found that Ca inhibition of the Na/K pump mediated Rb influx needs to take into account Mg_c levels. Furthermore, the Ca_c inhibition is fully reversible by increasing Mg_c suggesting reversible competition of Ca and Mg_c for ATP, and other sites for which Ca_c may have even a higher affinity (Beauge and Campos, 1983). The uncompetitive inhibition of ouabain-sensitive Rb pump influx suggests that Ca_c indeed may act at the E_2K form, i.e. at the K occluded intermediate of the cycle. Beauge and Campos (1983) also found that Ca increased $K_{0.5}$ for K in the Na/K ATPase, and discussed the possibility of Ca effected K dissocclusion. Furthermore, Ca inhibited catalysis of phosphorylation from P_i by Mg (Post and Stewart 1984) and the K-stimulated K-NPPase through the Na/K ATPase by simple competition with Mg (Rossi et al. 1978; Robinson 1974). Also, Ca competes at the monovalent K binding center (Vasallo and Post 1986). Further work is needed to see whether the pumps of HK and LK sheep red cells respond differently at the proposed level of Ca_c action.

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CHARGE TRANSFER BY THE Na/K PUMP

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INTRODUCTION

This report summarizes observations on effects of membrane potentials on the pump and measurements of electrogenic potentials. These provide information on mechanism and identity of charge carrying steps in the cycle. This work utilizes phospholipid vesicles reconstituted with renal Na/K ATPase, a system which shows several important features for these purposes including (a) simplicity (b) control of sidedness of ligand effects (c) accessibility of the cytoplasmic surface to the medium and (d) a very low passive cation permeability or electrical conductance.

VOLTAGE-SENSITIVITY OF THE Na/K PUMP

The Na/K pump is electrogenic and its rate must therefore be sensitive to voltage. The basis for the analysis of voltage effects is that the rates of steps carrying charge through the electric field should be sensitive to membrane potential. These charges include the ions themselves, groups on the protein to which the ion bind or movement of charges and displacement of dipoles unrelated directly to the transport. From observations on effects of voltage on the steady-state transport rate or pump current and especially on transient phenomena reflecting isolated steps, it is possible in principle to identify charge carrying steps, deduce the number of charges moving and the dielectric distance through which the charge moves (Lauger and Apell, 1986). The most likely candidates for the charge carrying steps are the transport linked conformational changes and/or ion binding or dissociation.

The rich mechanistic information contained within the voltage dependence of the pump has made this subject a focus of intense interest recently. Effects of voltage have now been observed in several whole cells particularly heart cells (Gadsby et al., 1985; Nakao and Gadsby, 1986a), on the isolated renal (Na,K)ATPase reconstituted into phospholipid vesicles (Goldschleger et al., 1987a), and in addition, transient currents generated by Na/K pump have been recorded from membrane fragments bound to planar bilayers (Fendler et al., 1985; Borlinghaus et al., 1987; Apell et al. 1987). The outcome of these efforts is a unified and relatively simple picture of the charge carrying steps, essentially consistent with the model proposed at Cambridge in 1984 (Karlish et al., 1985; see De Weer et al., 1987).

In reconstituted vesicles diffusion potentials, negative inside, are generated with K-gradients plus valinomycin or Li-gradients plus a Li-ionophore AS701. We have been able to test for and detect effects on active Na/K exchange and Rb/Rb exchange, and directly on conformational transitions using reconstituted fluorescein-labelled (Na,K)ATPase.

1. The initial finding was that at saturating cytoplasmic Na and ATP concentrations, the membrane potential -150-200 mV inside, accelerated by 30-40% ATP-dependent Na/K exchange on inside-out oriented pumps, but the effect disappeared at rate-limiting ATP concentration (1 μM). Because the conformational transition $E_2(2K) \rightarrow E_1 2K$ associated with transport of $2K^+$ ions, is known to be very rate-limiting at low ATP we concluded that this transition is a voltage-insensitive step. Conversely at saturating ATP the conformational transition $E_1P(3Na) \rightarrow E_2P3Na$ associated with transport of 3Na ions is partially rate-limiting and this transition was therefore deduced to be a voltage-sensitive transition. Phosphorylation and dephosphorylation rates were not considered rate-limiting.

2. The accelerating effect of the potential is greater at limitingly low cytoplasmic Na than at saturating concentration, an effect expressed as an increase in apparent Na affinity and decrease in Hill coefficient for Na activation curves.

3. (ATP+Pi) activated Rb/Rb exchange was not affected by the membrane potential, providing independent evidence that K transport steps are voltage-insensitive.

4. Direct confirmation of the assignment of charge carrying steps has now been obtained. Fluorescein-labelled (Na,K)ATPase was reconstituted into vesicles, and E_1 -- $E_2(K)$ conformational transitions on inside-out oriented pumps could be recorded by the Na- or K-induced fluorescence changes. As expected diffusion potentials affected neither the rates of $E_2(K) \rightarrow E_1K$ and $E_1K \rightarrow E_2(K)$ nor the concentrations of Ka or Na required to induce

the conformational transitions (Rephaeli et al, 1986a). Fluorescein-labelled (Na,K)ATPase does not bind ATP, precluding observation of fluorescence signals associated with turnover. However, following a report that acetyl phosphate activates Na transport in vesicles (Beauge et al., 1985) we have found that the acetyl phosphate acts as a substitute substrate on FITC-enzyme, and in the presence of Na and Mg fluorescence quenching was observed. This was attributed to the steps $E_1 \rightarrow E_1P \rightarrow E_2P$. The rate of this fluorescence signal is a measure of E_1P (3Na) $\rightarrow E_2\tilde{P}$ in our conditions, and as predicted was accelerated by the diffusion potential, -173 mV producing up to a four-fold effect (Rephaeli et al., 1986b).

CONCLUSION

1. The simplest model to explain voltage-insensitivity of K-transport and voltage-sensitivity of Na-transport is that there are two negative charges in the cation binding domain (asp, glu?) and the net charge movements when $2K^+$ or $3Na^+$ are transported are 0 and +1 respectively. Furthermore the simplest assumption would be that there are no net charge movements in the field other than those of the sites with their bound cations and also that 2K ions are bound to the same negatively charged residues as are 2Na ions but the third Na ion is bound to a 'neutral' site.

2. A kinetic analysis reveals that the effect of voltage on apparent Na affinity is due to an increase in an intrinsic Na binding affinity. This could be explained by (a) the existence of a shallow Na well, of depth about 0.1 of the dielectric distance or (b) a direct effect of potential on protein conformation and thus intrinsic cation affinity.

ELECTROGENIC POTENTIALS

Potentials generated by the Na/K pump have been detected in many whole cells, particularly when Na-loaded. The magnitude of the electrogenic potential should equal the product of the pump current (proportional to the pump density) multiplied by the resistance per unit area. The resistance of most cell membranes is such that electrogenic potentials rarely exceed 10 mV. In red cells too, the Na/K pump is electrogenic, measurements with a carbocyanine dye giving values in the range of 2-10 mV (Hoffman et al., 1979). Here the very low pump density is offset by deliberately raising membrane resistance to $10^6\ \Omega/cm^2$ with DIDS, the inhibitor of the anion transporter. An important result in DIDS-treated red cells is that, the 'uncoupled' Na efflux into Na and K free medium is electroneutral. The finding of a ouabain-sensitive sulfate efflux lead Dissing and Hoffman (1983) to propose that in Na and K free media the pump can

sustain an electroneutral Na/anion co-transport. This suggestion was sufficiently provocative to make it worthwhile testing it in a different experimental system.

With reconstituted vesicles loaded with K, addition of ATP to a medium containing Na plus Mg, activates Na/K exchange on inside-out oriented pumps and should generate a potential positive inside. We have now measured this ATP-induced potential by detecting absorption changes at 628-590 nmetre of a lipophilic anionic dye Oxonol VI, using a DW2 double-beam spectrophotometer (Goldschleger et al., 1987b). The absorption changes are produced by the shift in the absorption spectrum accompanying accumulation of the dye. The magnitude of electrogenic potentials has been calibrated with artificial diffusion potentials generated with pH gradients plus a protonophore. The following summarises results and interpretations of signals accompanying normal and abnormal flux modes.

1. ATP-dependent Na/K exchange. Very large absorption changes accompany Na/K exchange. The steady-state voltage is attained when the pump current equals the passive leakage currents. Calibration of the absorption changes shows that, routinely, potentials of 100-200 mV, and sometimes as high as 300 mV, can be generated in conditions of maximal Na/K exchange. These large potentials obviously reflect on the high electrical resistance of the lipid. It is found that the curves for activation of the oxonol signal by ATP and Na_{cyt} over wide concentration ranges, parallel closely the curves for activation of the ATP dependent ^{22}Na flux into K-containing vesicles. These findings indicate that the electrogenic potential is proportional to the pump current as expected and the pumping stoichiometry of 3 $Na_{cyt}/2K_{exc}$ must be constant and fixed over wide ranges of ATP and Na_{cyt} concentrations.

2. (ATP+Pi)-dependent K/K(Rb) exchange (rate $\sim$ 15% of Na/K exchange). This flux is electroneutral presumably with a stoichiometry $2K_{cyt}/2K_{exc}$.

3. ATP-dependent Na/Na exchange (rate $\sim$ 3% of Na/K exchange). This flux generates a signal 0.5-0.6% of that generated by Na/K exchange. This exchange flux is thought to occur with a stoichiometry of 3 $Na_{cyt}/2$ Na_{exc}, 2 Na_{exc} acting at K-sites The present result would suggest that some cycles of exchange are indeed electrogenic.

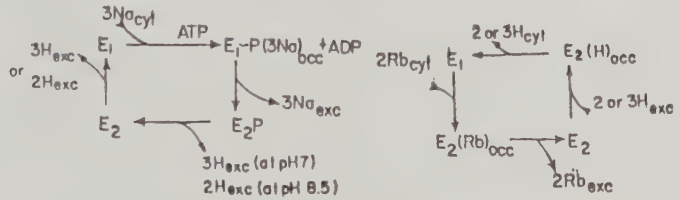
3. ATP-dependent Na uptake into Na- and K-free vesicles (rate $\sim$ 1% of Na/K exchange). At pH 7 this flux is essentially electroneutral in agreement with Dissing and Hoffman (1983). Electroneutrality could be consistent with either co-transport 3 $Na_{cyt}/3$ Cl_{exc} as proposed or exchange 3 $Na_{cyt}/3$ H_{exc}.

Unexpectedly, when the pH of the medium is raised the 'uncoupled' flux becomes electrogenic, with an optimum at pH 8.5. Quantitative measurements suggest that the ion stoichiometries in the 'uncoupled' and Na/K exchange modes are equal at at pH 8.5 (e.g. 3 $Na_{cyt}/2\ Cl_{cyt}$ or 3 $Na_{cyt}/2\ H_{exc}$ in the 'uncoupled' mode). The rate of ATP-dependent ^{22}Na flux was found to be quite independent of the anion in the medium for the series, Cl, NO_3, formate, aspartate and gluconate. The appearance of electrogenicity when the proton concentration was reduced and lack of anion specificity are obviously suggestive of Na/H exchange, i.e. 3 $Na_{cyt}/3\ H_{exc}$ at pH 7.0 and perhaps 3 $Na_{cyt}/2\ H_{exc}$ at pH 8.5

5. Vanadate-Inhibited Net K(Rb) movement into K and Na-free vesicles (rate 0.3-0.5% of maximal Na/K exchange). Characteristics of the slow net passive 'slippage' flux of K, and similar exchange fluxes of K with other monovalent cations, have been described in detail (Karlish and Stein, 1982). No oxonol signal accompanies the net Rb flux into Na and K-free vesicles and it is probably therefore electroneutral. In addition the Rb flux shows the same lack of anion specificity as does the 'uncoupled' Na flux. Hence we suggest that this flux too is in reality an exchange $2Rb_{cyt}/2H_{exc}$.

A MODEL

The following models represent the sodium/proton or potassium/proton exchanges suggested to occur in the absence of Na or K at the extracellular surface. This hypothesis mirrors the finding that the pump sustains an ATP-dependent H_{cyt}/K_{exc} exchange in the absence of Na at the cytoplasmic surface (Hara and Nakao, 1986).



An important implication of this hypothesis, if correct is that the sites must bind protons at pH 7.0. SInce as concluded above it is apparently carboxyl groups which bind the ions (Na, K or H) the pK's of these residues must be appreciably higher than in free solution (pH 4-5). Hence it is likely that upon binding ions the relevant carboxyl's are shielded from the aqueous medium e.g. by transfer to the intra membrane domain.

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THE VECTORIAL EFFECT OF LIGANDS ON THE OCCLUDED INTERMEDIATE IN RED CELL SODIUM PUMP TRANSPORT

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INTRODUCTION

In 1972, Post and coworkers demonstrated the existence of an occluded cation form of the sodium pump that was produced following dephosphorylation by K^+. ATP, acting with a low affinity, accelerated the release of this cation (Post et al., 1972), indicating that ATP was able to activate the sodium pump in a fashion that was functionally different from its action as a phosphate donor. It was later suggested by Karlish et al. (1978), that the occluded enzyme (represented here as $E_2(K)$) was the predominant form in Na-free, K containing media and measurements on the decrease in the intrinsic tryptophan fluorescence of purified enzyme from pig kidney revealed that the conversion from $E_2(K) \longrightarrow E_1Na$ was slow ($K = 0.25\ s^{-1}$ at 20^o C, Karlish & Yates, 1978). This conversion was accelerated by nucleotides acting with low affinity in the absence of phosphorylation or hydrolysis.

Direct experimental demonstration of the occluded enzyme was first reported by Beauge & Glynn (1979) using a cation exchange column and more recently by Forbush measuring ^{86}Rb dissociation rates with a rapid filtration apparatus (1984). Direct exposure of the enzyme to ADP, ATP or P_i leads to a rapid release of ^{86}Rb (Glynn & Richards, 1982, Forbush, 1984, 1987). The assumption in

these experiments is that ATP (or ADP) stimulates ^{86}Rb release only to the inside and P_i only stimulates release of this cation to the outside. Since these results were obtained from preparations of purified enzyme, where sidedness has been removed, it is of interest to examine the effects of these ligands on transport reactions in red blood cells where sidedness is maintained and the pump is in its native state in the membrane.

METHODS

Resealed ghosts were prepared using a gel filtration column previously described (Kaplan, 1982, Kenney & Kaplan, 1987), based on a procedure designed by Wood and Passow (1981). ^{86}Rb uptake measurements were a modification of a method previously described (Kaplan & Kenney, 1982).

RESULTS

We first prepared resealed ghosts from human red cells that contained various cations, anions and intracellular pH buffers only and measured the ouabain-sensitive ^{86}Rb uptake. In the absence of nucleotides or phosphate, this Rb:Rb exchange represents about 1% of the maximum Na + K-stimulated pump flux in red blood cells (Garay & Garrahan, 1973), but is still readily measureable. These small "passive fluxes" have been previously observed in phospholipid vesicles containing purified enzyme from pig kidney reconstituted using a freeze-thaw sonication procedure (Karlish & Stein, 1982).

We then determined the effect of several K congeners in stimulating sodium pump-mediated ^{86}Rb uptake (Table I).

Table I

THE EFFECT OF DIFFERENT INTERNAL CATIONS ON PASSIVE ^{86}Rb UPTAKE

INTERNAL CATION 50 mM	- OUABAIN (μmol Rb^+ $Lcells^{-1}$ hr^{-1})	+ OUABAIN	SODIUM PUMP COMPONENT
Rb	42.55 ± 5.93	25.04 ± 3.59	17.51
K	51.73 ± 5.59	30.08 ± 4.60	21.65
Na	18.51 ± 2.36	18.96 ± 3.82	0.0
Li	37.95 ± 3.24	20.17 ± 3.82	17.78
Cs	34.62 ± 3.24	11.95 ± 1.98	22.67
Cho	19.97 ± 2.31	19.43 ± 2.99	0.54

Cs = K > Li = Rb > > > > Cho = Na

As expected, K, Rb, and Cs all supported Rb:X exchange, Na or choline did not. These data suggest that in passive Rb uptake, the sodium pump functions as a simple facilitated diffusion system and when intracellular Na or choline are present, the return of the empty carrier does not occur. Surprisingly, Li, previously shown to be an intracellular Na substitute (Rodland & Dunham, 1980), also supported ouabain-sensitive ^{86}Rb uptake, when Na did not.

Previous work on the role of K in dephosphorylating the enzyme reported that the sequence for cation-stimulated rephosphorylation is Rb > K > Cs > Li, indicating that Li is less tightly bound by the enzyme (Post et al., 1972). More recently, experiments testing the direct effect of these cations in stimulating release of ^{86}Rb bound to purified enzyme has confirmed this sequence (Forbush, 1987). This observation, together with our observation that Li would promote passive Rb uptake (Table I), allowed us to devise the following experiments to investigate the role of P_i and ADP (ligands which should vectorially stimulate deocclusion) in an intact, sided system under transporting conditions.

The experimental design is shown in Scheme 1. Ghosts were prepared to contain Rb or Li (50 mM) and the effect of ADP or P_i on ouabain sensitive ^{86}Rb uptake was determined (Table 2). When Rb is the internal cation, the expectation is that both ligands (ADP and P_i) will stimulate Rb:Rb exchange, since Rb is tightly occluded by the enzyme. However, when Li is the intracellular cation, only ADP should stimulate ^{86}Rb uptake (by stimulating release of labeled Rb to the inside) and P_i should have virtually no effect in stimulating Li release to the outside, since it is much less tightly bound. As shown in Table 2, the results are as expected.

SCHEME I

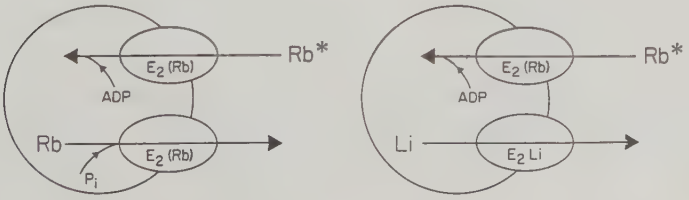

TABLE II

Internal Cation	Ouabain-sensitive ^{86}Rb Uptake ($\mu mol\ Rb^{+}1.cells^{-1}h^{-1}$)		
50 mM	0 ADP, 0 P_i	+ 1 mM ADP	+10 mM P_i
Rb	9.8	136.0	34.5
Cs	23.5	131.2	51.5
Li	12.5	50.0	16.0

The other type of experiment involves the ATP release of K inhibition observed in the presence of low (uM) levels of ATP (shown in Scheme 2). When [ATP] is low, K inhibits the Na-ATPase activity (presumably as the enzyme builds up in the $E_2(K)$ form) (Czerwinski et al., 1967, Neufeld & Levy, 1969, Blostein, 1970) and this inhibition is removed when higher concentrations (mM) of ATP or ADP are added (Post et al., 1972, Mone & Kaplan, 1982). Consequently, the efflux of ^{22}Na from resealed ghosts into Rb-containing medium should be low when ATP is 50 uM and stimulated when the [ATP] is increased. When Li is present as the external cation, the enzyme will not accumulate in the occluded form and raising the [ATP] should not further increase the flux. The results are shown in Table 3. It is worth noting that the rate of Li_o-stimulated ^{22}Na efflux is greater (about 2-fold) in the presence of the lowest [ATP] compared to the flux in the presence of 2 mM Rb_o.

SCHEME II

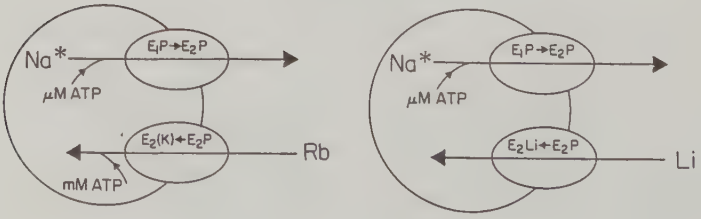

TABLE III

Ouabain-sensitive ^{22}Na Efflux

(mmol Na^+ $l.cells^{-1}h^{-1}$)

External Cation	50 uM ATP	5 mM ATP
2 mM Rb	0.98	4.02
50 mM Li	2.03	2.00

CONCLUSIONS

These data suggest that during Rb:Rb exchange, ADP speeds release of the occcluded cation only to the inside and phosphate stimulates release of the cation only to the outside. When the cation is not tightly occluded, as when Rb is replaced by Li, these ligands are without effect. Lastly, when the efflux of Na is coupled to the entry of a cation, the flux is not further stimulated by high ATP concentrations if the entering cation is not tightly occluded.

ACKNOWLEDGEMENTS

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PROPERTIES OF TWO TYPES OF HELA LOW K^+ (LK^+) MUTANTS WITH RESPECT TO K^+ TRANSPORT

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INTRODUCTION

In mammalian cells the intracellular potassium concentration is between 130-180 mM compared to 5-7 mM potassium in the culture medium, while intracellular sodium concentration ranges from 15 to 40 mM in contrast to approximately 150 mM extracellular sodium in MEM (minimal essential medium). These ion gradients across the cell membrane are generated and maintained by the Na^+,K^+ -ATPase and other transport proteins. One of these other proteins, transporting $Na^+,K^+,2Cl^-$, is the loop diuretic sensitive cotransporter, which is apparently present in all mammalian cells (Frizell et al., 1979; Gerencser and Lee, 1983). The $Na^+,K^+,2Cl^-$ -cotransport system has been suggested to function in volume regulation as previously described by Geck et al. (1981, 1983), or Cl^--transport (Greger and Schlatter, 1981), but its actual function has not been established yet. It was previously shown by Boardman et al. (1974); Pollack et al. (1982), that HeLa cells , when grown in a medium containing 0.5 mM potassium, respond to the lowered K^+ concentration by up regulation of functional Na^+,K^+ -ATPase molecules. In medium with 0.5 mM K^+ an up regulation of the Na^+,K^+ -ATPase is apparently sufficient to maintain cation gradients and enable the cells to proliferate. However, upon further reduction of the extracellular potassium concentration below a certain threshold-level (0.4 mM K^+) an increased number of Na^+,K^+ -ATPase molecules/cell alone is insufficient for proliferation of the cells. To investigate which transport processes limit the ability of HeLa cells to survive and proliferate in medium with 0.2 mM K^+, we mutagenized HeLa cells and selected them for growth in medium with 0.2 mM potassium. Some of the mutants obtained, were used for the transport studies descrided here.

METHODS

In all experiments HeLa monolayer cells were used. The parental cells were kept in Joklik's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS). The mutants (LK^+_7, LK^+_{11}) were kept in MEM suppl. with 10% dialysed FCS and a final potassium concentration of 0.2 mM. Cells were mutagenized with 400 µmol/l ethylmethane sulfonic acid (EMS) in MEM for 20 h. Then, the cells were selected for growth in 0.2 mM K^+ MEM. Two (LK^+_7 and LK^+_{11}) of the numerous clones, which were obtained, were utilized for further studies. For the experiments described below, cells were trypsinized from 10 cm plates and seeded two days before testing into 16mm wells of 24 well plastic tissue culture trays (Nunc). All measurements were done with cells at a density of approx. $5x10^5$ cells/ml.

$^{86}Rb^+$ Influx Measurements

The cells in the Nunc plates were washed twice with phosphate-buffered saline (PBS, which contains: 135 mM NaCl, 6.5 mM Na_2HP0_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 5 mM Glucose, O.7 mM $CaCl_2$ and O.5 mM $MgCl_2$), at 37°C and a final pH of 7.2. Then they were preincubated for 10 min with PBS at 37°C in a water bath shaker. The buffer was removed and replaced with the test solution which contains $^{86}Rb^+$ as a tracer of potassium movements. The influx was measured for a period of 10 minutes (during which uptake is essentially linear) in PBS at 37°C, pH 7.2, containing 2 µCi $^{86}Rb^+$/ml. Experiments were terminated by aspiration of the solution and the cells were rapidly rinsed two times, with 500 µl PBS to remove extracellular radioactivity. Cells were solubilized in 0.2% sodium-dodecylsulphate (SDS) and radioactivity was determined, using a Packard autogamma scintillation spectrometer CP 153.

$^{86}Rb^+$ Efflux Measurements

Cells were washed two times with PBS and then preloaded with $^{86}Rb^+$ for 90 min in PBS, containing 2-3 µCi $^{86}Rb^+$/ml, at 37°C and 5% CO_2-atmosphere in an incubator. After aspiration of the solution the monolayers where rapidly washed twice and incubated with efflux buffer (= K^+ free PBS). At various time intervals efflux was terminated by using the same protocol as described in the $^{86}Rb^+$ influx experiments. Cell volume was determined as described by Lubin (1980). Protein

was determined by the tryptophan fluorescence method as described by Avruch and Wallach (1971), using a Perkin elmer fluorescence spectrophotometer 650-50 with slit widths 4/10 nm, excitation wavelength 284 nm and emission wavelength 325 nm. K^+ contents of logarithmically growing cells were determined as following: The medium was aspirated and the cells were rapidly washed 6 times with ice-cold solution, which contained 100mM $MgCl_2$. Then the cells were solubilized by adding 1 ml of 5% perchloric acid to a well and potassium released, was determined with an Eppendorf flame-photometer.

Determination of ^{3}H-Ouabain Binding Sites

The medium was aspirated and the cells were washed twice with K^+ free PBS and then incubated with K^+ free PBS, containing various concentrations of ^{3}H-ouabain, ranging from $5x10^{-9}$ M to $1x10^{-7}$ M, at 37°C. Nonspecific binding was determined in parallel samples by adding unlabeled ouabain at a conc. of $1x10^{-3}$ M, and $2.5x10^{-8}$ M ^{3}H-ouabain to the test buffer. The incubation was terminated by aspiration of the solution and washing the cells three times with K^+ free PBS at 37°C. Finally the cells were solubilized and radioactivity was counted in a Packard tri carb 4640 liquid scintillation counter. The number of ^{3}H-ouabain binding sites was determined from Scatchard plots, as described by Scatchard (1949).

All data given, are the mean of at least 4 experiments. Significance was calculated by a students t-test.

RESULTS

The results of a comparative characterisation of some basic properties of HeLa cells and both LK^+-mutants are summarized in Table 1.

	HeLa parental	LK^+_7	LK^+_{11}
growth rate (i.e. doubling time)	20h*	45h**	50h**
mg protein/ 10^6cells	0.089*	0.119**	0.138**
rel. cell. vol. in %	100%*	134%**	155%**
intracellular K^+ in mM	160-180*	90-120**	95-115**

* Cells were kept with Joklik's MEM suppl. with 5% FCS.
** Cells were kept with low K^+MEM suppl. with 10% dialysed FCS.

$^{86}Rb^+$ Influx Measurements

When $^{86}Rb^+$ influx was measured in 4.2 mM K^+ PBS, both mutants (LK^+_7, LK^+_{11}) exhibited a higher total $^{86}Rb^+$ influx, as compared to the parental HeLa cells (LK^+_{11} 242%, LK^+_7 140%, parental cells 100%). The bumetanide sensitive influx is about 600% higher in the LK^+_{11} mutants and about 230% higher in the LK^+_7 mutants, as compared to the parental cells. When expressed as a fraction of the total influx in the respective mutants, the diuretic sensitive component was only increased about 20% in the LK^+_{11} cells and 10% in the LK^+_7 cells. Likewise the ouabain sensitive influx is increased 268% in the LK^+_{11} cells and 178% in the LK^+_7 mutants. The contribution of the Na^+,K^+ -ATPase to the total influx in the mutants is also increased, but only sligthly (LK^+_{11} 5%, LK^+_7 12%). A comparison between the parental cells and the mutants is shown in Fig 1.

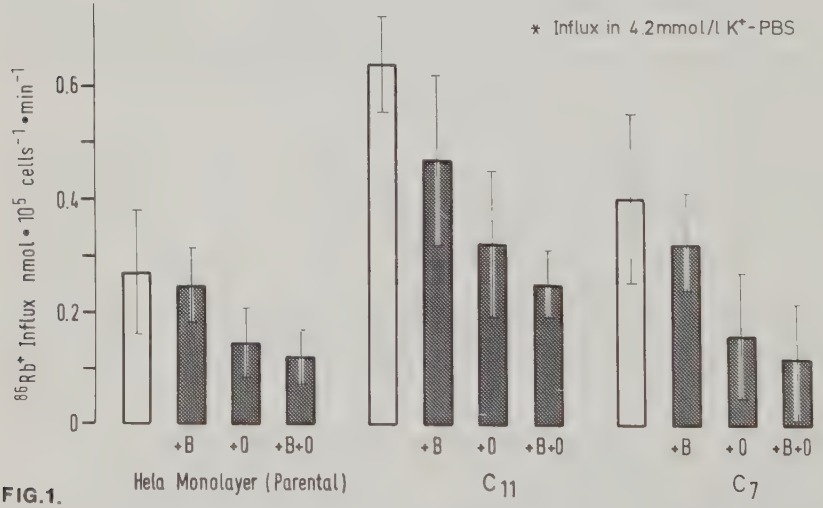
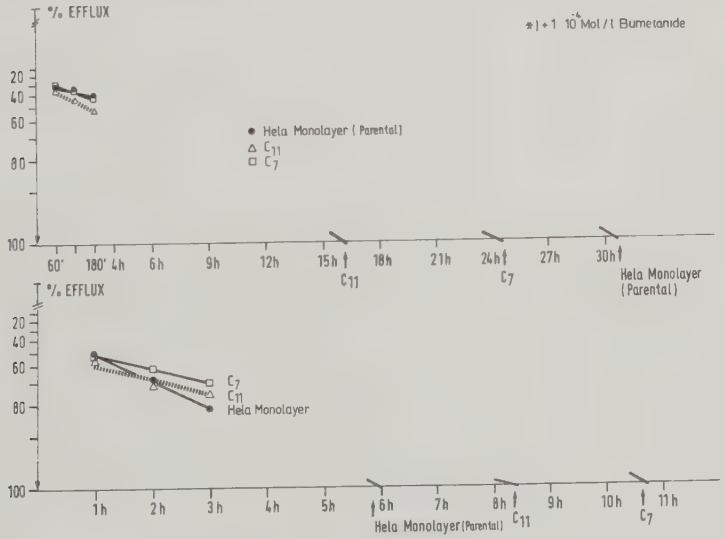


FIG.1.

$^{86}Rb^+$ Efflux Measurements

When plotted on a semilogarithmic scale two components of $^{86}Rb^+$ efflux are observed in the parental HeLas as well as in the mutants: A rapid efflux within minutes and a slower efflux component proceeding for hours. Similar findings were reported by Jayme et al. (1981, 1984) for mouse fibroblasts. There was no difference between the parental HeLa cells and the mutants, with respect to the fast component of efflux. In contrast, in both mutants the rate of the second component of efflux was much slower than in the parental cells, especially pronounced in the LK^+7 mutants , i.e. the mutants (LK^+7 and LK^+11) maintained higher intracellular $^{86}Rb^+$ concentration upon prolonged incubation in potassium free PBS. When the long term $^{86}Rb^+$ efflux is measured in the presence of bumetanide however, the parental cells maintained a higher intracellular $^{86}Rb^+$ concentration compared to both mutants (Fig. 2). Thus it appears, that the efflux via the $Na^+,K^+,2Cl^-$- cotransport system is reduced in both mutants, and hence the decrease of efflux in the mutants is due to a reduction of the bumetanide sensitive component of efflux under these experimental conditions.

Fig. 2.



^{3}H-Ouabain Binding Sites

The data of these experiments are given in table 2. After substraction of the nonspecific binding both mutants exhibited a 3-4 fold increase of ^{3}H-ouabain binding sites/cell, while grown on low K^+ MEM. The results from the determination correlate with the data of $^{86}Rb^+$ influx measurements. Compared to the parental HeLa cells the number of Na^+,K^+ -ATPase molecules/cell was maintained at a higher level in both mutants (range 150-170%), when grown with "normal" MEM.

Table 2.

	^{3}H-ouabain binding sites/cell
HeLa parental	$0.5 \cdot 10^{6}$*
LK^+_7	$1.8 \cdot 10^{6}$**
LK^+_{11}	$2.2 \cdot 10^{6}$**

The results of a preliminary kinetic characterisation of the cotransporter in the parental cells and in the mutants with respect to K^+ influx are summarized in Table 3.

	HeLa parental	LK^+_7	LK^+_{11}
K_m mM	5.7*	1.5**	4.0**
V_{max} nM/ 10^5cells·min.	0.1*	0.04**n.s.	0.20**

DISCUSSION

The mutants described in this report and especially the LK^+_{11} cells differ in several aspects from previously described mutants (Jayme et al., 1981, 1984). Both cell lines obtained here, exhibited elevated activities of of the Na^+,K^+ -ATPase and the $Na^+,K^+,2Cl^-$ -cotransport system, when grown on 0.2 mM K^+ MEM. With respect to the cotransporter both mutants showed an increase in the bumetanide sensitive influx component, but a decrease in the loop diuretic sensitive efflux fraction. The increased influx via the Na^+,K^+ -ATPase and the $Na^+,K^+,2Cl^-$ -cotransport system apparently is brought about by different means in the two mutants.

The LK^+_{11} cells have a higher number of Na^+,K^+ -ATPase molecules/cell and probably a higher V_{max} of the $Na^+,K^+,2Cl^-$ -cotransport system. If one accepts, that the V_{max} is a measure of the number of transport proteins, then there should be a higher number of $Na^+,K^+,2Cl^-$ -cotransport system molecules/cell. On the other hand, the LK^+_7 mutants exhibited a change in the K_m of the $Na^+,K^+,2Cl^-$ -cotransport system (Table 3), as well as an up regulation of the number of the Na^+,K^+ -ATPase molecules. However these kinetic data have to be interpreted with caution, since it is not known, whether they are "real V_{max} values", i.e. V_{max} values have to be measured in addition after maximal stimulation of the cotransporter, due to hypertonic treatment of the cells, and these data should be compared with the data obtained here. Nevertheless, our findings lead to the conclusions: (1) an up regulation of the Na^+,K^+ -ATPase alone is not sufficient for HeLa cells to survive at extracellular K^+ concentration below 0.4 mM. (2) The $Na^+,K^+,2Cl^-$ -cotransport system is involved in cation homöostasis and seems to play an important role in accumulation of potassium under these conditions. (3) The LK^+_7 cells partially resemble previously described mutants (Jayme et al., 1981, 1984) and their ability to survive in low K^+ medium is probably directly related to the reduced K^+ efflux. The LK^+_{11} cells represent a totally different type of LK^+ mutants. The potassium transport as a whole seems to be altered in these cells, with respect to all components of K^+ influx, accompanied by some reduction of K^+ efflux. The ability of the LK^+ cells to grow in low K^+ medium, is probably due to the increased K^+ uptake via the Na^+,K^+ -ATPase and the $Na^+,K^+,2Cl^-$ -cotransport system and is not related to a reduction in K^+ efflux. At present, we can only speculate about the mutation(s) enabling these cells to grow in low K^+ medium. Since both of the two K^+-transport systems studied, are affected, the mutation is either pleiotropic (i.e. a mutation acting on a regulatory mechanism, which affects the Na^+,K^+ -ATPase and the $Na^+,K^+,2Cl^-$ -cotransport system), or the two transport systems are amplified in parallel in these cells.

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TWO DIFFERENT TYPES OF ATP-DEPENDENT ANION COUPLED Na TRANSPORT ARE MEDIATED BY THE HUMAN RED BLOOD CELL AND Na/K PUMP

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INTRODUCTION

This paper summarizes recent work defining two distinct mechanisms by which the human red blood cell Na/K pump can transport anions. These kinds of anion transport are coupled to Na extension in a manner that contrasts sharply with the normal operation of the Na/K pump. Thus, anions do not seem to be involved in any evident (or measurable) way when the pump is electrogenically exchanging 3 Na_i (inside) for 2 K_o (outside) at the expense of 1 ATP (Post and Jolly, 1957; Sen and Post, 1964). However, this is not the case in uncoupled Na efflux, first described by Garrahan and Glynn (1967a,b). Uncoupled Na efflux refers to a pump mediated mode whereby Na efflux continues to take place in the absence of K_o and Na_o, where these ions can be substituted for by Choline$_o$, Tris$_o$ or Mg. Since there was no obvious external cation to exchange for Na_i, this flux mode was termed "uncoupled". This uncoupled Na_i efflux is ATP dependent and is inhibited by ouabain. Uncoupled Na efflux can also be inhibited by Na_o but the inhibition by Na_o (maximum at 5 mM) is always exceeded by ouabain. Higher concentrations of Na_o activate Na efflux presumably by stimulating Na/Na exchange and ATPase activity (cf. Glynn and Karlish, 1976; Blostein, 1983).

As already alluded to Na efflux, in the absence of K_o and Na_o, is not really "uncoupled" but occurs in conjunction with the cotransport of cellular anions. That anions are coupled to the flux of Na began with the finding that uncoupled Na efflux was electroneutral (Dissing and Hoffman, 1983). This was done by employing the same fluorescent dye technique that was used to establish that the normal functioning red cell Na/K pump is electrogenic (Hoffman et al., 1979). Thus, these studies were carried out with DIDS-treated intact cells (or resealed ghosts) in which all of the Cl (on both sides of the membrane) had been replaced and equilibrated with SO_4. DIDS was used in order to inhibit all anion exchange through Band 3 making it possible to measure the efflux of SO_4 (with $^{35}SO_4$) in concert with Na. Uncoupled Na efflux is unaffected in cells exposed to DIDS and/or SO_4 but SO_4 has the advantage that its base background flux is much lower than Cl in these circumstances.

EFFLUX OF CYTOPLASMIC ANIONS (SO_4)

The basic observation was that for the SO_4 efflux that was found to occur, this SO_4 efflux was completely inhibited by 5 mM Na_o and by ouabain. While this result was at first puzzling because ouabain-sensitive Na efflux exceeded (by about 10 to 20%) 5 mM Na_o-sensitive Na efflux, it should become evident, as discussed below, that this residual Na_o-insensitive, ouabain-sensitive Na efflux is coupled to a different type of anion efflux. In any case, the stoichiometry of the Na_o-sensitive Na efflux was twice that of the Na_o-sensitive SO_4 efflux, consistent with the finding that this coupled efflux was electroneutral, transporting 2 Na_i^+ per $SO_{4i}^=$. The SO_4 efflux, like Na efflux, varied with the concentration of Na_i and was dependent on ATP. It was also possible to pre-equilibrate varying concentrations of Cl across the membrane together with SO_4 prior to exposure to DIDS. As mentioned before, varying ratios of Cl/SO_4 had no effect on Na efflux, but as little as 5 mM Cl_i in the presence of 45 mM SO_{4i} eliminated Na_o-sensitive SO_4 efflux. Evidently Cl_i is the anion of preference for the electroneutral efflux of Na.

If anions are cotransported with Na in uncoupled Na efflux, then how is Na efflux affected when internal SO_4 is switched to a relatively impermeant anion, such as

tartrate? To test this effect, hemoglobin-free ghosts were prepared to contain an equilibrated concentration of tartrate as the principal anion together with Na, Choline, Mg and ATP prior to being resealed and DIDS-treated. Two important results emerged from these kinds of experiments. The first was that uncoupled Na efflux was markedly inhibited by tartrate substitution and that this inhibition was completely reversed by the inclusion of 5 mM Cl_i prior to the treatment with DIDS. The Na pump also became slightly electrogenic in the tartrate-loaded ghosts but not when the ghosts also contained 5 mM Cl_i. These results are consistent with the idea that Na and anions are cotransported in uncoupled Na efflux because Na efflux is inhibited in the absence of a permeable anion.

The second type result was that when K_o was added to tartrate-loaded ghosts, thereby activating the pumped exchange of Na_i for K_o, the Na/K pump became markedly electrogenic in the presence or absence of 5 mM Cl_i. The fact that the pump operated the same independent of the type of anion and its permeability indicates that anions are not involved in normal Na_i/K_o exchange. On the other hand, a problem is raised by the finding that when DIDS-treated, SO_4-loaded cells carry out normal electrogenic pump mediated Na_i/K_o exchange, that there is no measurable ouabain-sensitive SO_4 efflux. This is one way of indicating that the molecular basis for the pump's electrogenicity and the change in its charge structure that accommodates uncoupled Na efflux remains unknown at the present time.

EFFLUX OF SUBSTRATE ANIONS (PO_4)

The fact that there is a substrate source as well as a cytoplasmic source of anions cotransported with Na emerged from an analysis of the Na_o-insensitive ouabain-sensitive component of Na efflux. Two types of preparations were studied: one was intact red cells while the other was resealed ghosts prepared to contain [γ-^{32}P]ATP as specified by Glynn and Karlish (1976). Both were DIDS-treated and the results on PO_4 efflux were the same whether the principal cytoplasmic anion was Cl or SO_4. PO_4 efflux was measured from intact cells by determining the rate of appearance of ortho-PO_4 by the method of Forbush (1983). The results with intact cells showed that not only was there a ouabain-sensitive efflux of PO_4, but that this PO_4

efflux was unaffected by Na_o (from 5 to 150 mM). However, the PO_4 efflux was completely inhibited by 5 mM K_o (half-maximal at 0.3-0.4 mM K_o) independent of the presence of Na_o. Trapping 5 mM ortho-PO_4 inside intact cells prior to DIDS-treatment had no effect on the PO_4 efflux. PO_4 efflux changed in accordance with changes in Na_i such that the stoichiometry of cotransport was always constant at approximately 2 Na per PO_4. The cytoplasmic flux of SO_4 was about four times the flux of PO_4 and this ratio remained constant regardless of changes in the cotransport rate as altered by changes in Na_i.

The experiments with ghosts containing [γ-^{32}P]ATP showed that the PO_4 released (in this case $^{32}PO_4$) came from the γ-$^{32}PO_4$ of ATP. This is why we have specified substrate as the source of anion cotransported with Na for the Na_o-insensitive component of uncoupled Na efflux. Thus ghosts loaded with (or suspended in) non-radioactive ortho-PO_4 did not alter the ouabain-sensitive $^{32}PO_4$ efflux from incorporated [γ-^{32}P]ATP indicating that the transport of PO_4 was substrate derived and independent of bulk ortho-PO_4. In addition, when the situation was reversed by using ^{32}P labelled ortho-PO_4 and non-radioactive ATP, there was no ouabain-sensitive efflux of $^{32}PO_4$. Presumably that since there is no commingling of the γ-PO_4 from ATP and cytoplasmic PO_4, the $^{32}PO_4$ extruded by the pump in the uncoupled mode represents the dephosphorylation of the phosphointermediate associated with the Na_o-insensitive component of the Na efflux.

It should be emphasized that there is a paradox that underlies the above described results on the cotransport by the pump of anions together with Na. And this is that under all circumstances (in ghosts or in intact cells) the measured ratio of Na ions transported per ATP hydrolyzed in uncoupled Na efflux is constant at 3 Na per ATP. It is not obvious how two presumably electroneutral processes with balanced transport stoichiometries (2 Na/SO_4 and 2 Na/PO_4) can be integrated with the metabolic stoichiometry. In addition, it is also not clear what regulates the ratio of SO_4 to PO_4 transport as well as what controls the level of PO_4 transport itself since there is considerably more PO_4 hydrolyzed than is effluxed. It should also be mentioned that the valence of the transported PO_4 is not known.

CONCLUSIONS

It should be clear from the above described results that, in human red blood cells, anions are cotransported

with Na in uncoupled Na efflux. Two types of anion cotransport can be distinguished on the basis of the source of the anion transported. In one type, the cotransported anion comes from the cytoplasm (e.g. SO_4); in the other type, the cotransported anion (PO_4) comes directly from the pump's substrate, ATP. These types of cotransport are distinct in their sensitivity to Na_o. The SO_4 type is inhibited by Na_o whereas the PO_4 type is insensitive to Na_o. These different features imply that the red cell Na/K pump is actually made up of two distinct kinds that become evident upon conversion of the pump to its uncoupled mode of operation.

Mention should also be made of the roles Na_o and K_o play in specifying which transport reaction the Na/K pump should carry out. In this sense, Na_o must signal the pump to change its uncoupled efflux mode of the SO_4 type to Na/Na exchange, provided there is ADP available on the inside (Glynn and Hoffman, 1971). This means that Na_o must occupy at least one external site (not necessarily a transport site) that results in inhibition of SO_4 transport while providing for activation of Na influx. K_o, on the other hand, signals the inhibition of all anion efflux in converting the uncoupled flux mode to Na_i/K_o exchange. Presumably the K_o and Na_o bind to separate sites, but the degree of overlap is not known (see Kennedy, et al., 1986; Sachs, 1987).

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INTERACTION OF EXTERNAL Na^+ WITH THE Na^+-ATPase. A BY-PRODUCT INHIBITION?

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INTRODUCTION

In intact red blood cells the apparent first order rate constant for Na^+ efflux is reduced in the presence of very low external Na^+ concentration and this inhibition is reversed when the Na_o^+ concentration is increased between 5-100 mM (Sachs, 1970; Garrahan and Glynn, 1967). Glynn and Karlish (1976), in resealed red cell ghosts, and Lee and Blostein (1980), in inside out red blood cell vesicles, showed that increasing the external Na^+ concentration first inhibits the Na-ATPase activity with high affinity and then stimulates at Na^+ concentration higher than 5 mM. In broken membrane preparations and in the absence of K_o^+, the activity curve rises at low Na^+ concentration, reaches a plateau or slightly decreases between 3 and 10 mM Na^+ concentration and then continues to rise with higher Na^+ levels (Post et al., 1972; Beauge and Glynn, 1979).

High affinity Na^+ inhibition is reduced or totally abolished in enzyme preparations that have been previously treated with either n-ethylmaleimide (NEM) or oligomycin (Beauge and Glynn, 1979; Fahn et al., 1966). Since NEM and oligomycin reduce the rate of the transformation of ADP-sensitive to K^+-sensitive phosphoenzymes (Glynn and Hoffman, 1971; Albers, 1967; Yamaguchi and Tonomura, 1980), external Na^+ must inhibit after E_2P is formed in the normal kinetic cycle of the enzyme.

Beauge and Glynn (1979) found that 5 mM Na^+ strongly inhibits the dephosphorylation rate of pig kidney Na,K-

ATPase in K^+-free medium and have proposed this as the mechanism by which external Na^+ inhibits ATP hydrolysis. Although alternative possibilities have been proposed (Norby et al., 1983; Beauge, 1983) it still is not clear at which site (or sites) external Na^+ binds to produce the inhibition. This communication presents a model that qualitatively accounts for the effects of external Na^+.

RESULTS

A modification of the Post-Albers scheme is shown in Fig. 1. Magnesium has not been included and it is assumed to be present at saturating concentration. The enzyme is phosphorylated after the random binding of ATP and Na^+. Three Na_i^+ ions cooperatively bind to the enzyme (Foster and Ahmed, 1976) and, after phophorylation, are released to the exterior medium during the transformation $E_1P(Na^+)_3 \rightarrow E_2P$. The E_2P form could either release P_i or the dephosphorylation could be stimulated by binding of K_0^+ or Na_0^+ (Na^+ acting as a K^+-congener).

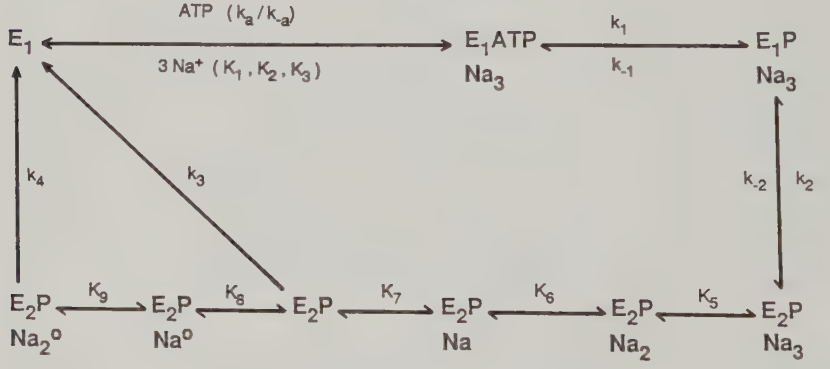

Figure 1. Scheme of the ATP hydrolysis carried out by the Na-ATPase.

The steady-state equations for enzyme activity were obtained using the methods of King and Altman (Cleland, 1967) and Cha (1968).

All the kinetic constants required for the calculation of the velocity are not currently available. Those taken from the literature were standardized to 37 °C using a Q_{10} of 2.7, assuming that the implicated process has a temperature coefficient equal to that of the overall Na,K-ATPase (Glynn et al., 1984). Table 1 shows the kinetic constants used in the simulation of the model. The dissociation constants of internal Na^+, K_1, K_2 and K_3, were taken from Foster and Ahmed (1976). The rate constants k_a and k_{-a} for binding and releasing, respectively, of ATP were taken from the data for formycin nucleotides, determined by Karlish et al (1978).

TABLE 1.Constants used to calculate the velocity equations.

Dissociation constants (mM)		Rate constants	
K_1	40.00	k_a	23000 s^{-1} mM^{-1}
K_2	3.40	k_1	500 s^{-1}
K_3	0.29	k_2	1200 s^{-1}
K_5	1000.00	k_3	8 s^{-1}
K_6	50.00	k_4	16 s^{-1}
K_7	0.50	k_{-a}	46 s^{-1}
K_8	30.00	k_{-1}	10000 s^{-1} mM^{-1}
K_9.	0.50	k_{-2}	2000 s^{-1}

Mardh and Post (1977) found that the pseudo first order rate constant for phosphorylation of guinea-pig kidney cortex Na,K-ATPase at 25 °C was about 200 s^{-1}. So, a figure of about 500 s^{-1} is obtained as a minimum value for k_1. The rate constant for the conversion from $E_1PNa^+_3$ to $E_2PNa^+_3$ was

calculated in the same way from Erohlich et al. (1976). A turnover number of about 150 s^{-1} was determined for the Na,K-ATPase at 37 °C (Jorgensen, 1980; Smith et al., 1980). At high Na^+ concentration, the enzyme activity in the absence of K^+ is 20-25 times less (Pedemonte and Beauge, 1986; Plesner and Plesner, 1981); i.e., about 7.5 s^{-1}. No first order rate constant in the ATP-hydrolysis cycle can be lower than this value. Thus, a value of 8 s^{-1} was assigned to the limiting rate constant velocity, k_3. All other dissociation and rate constants were obtained by trial and error.

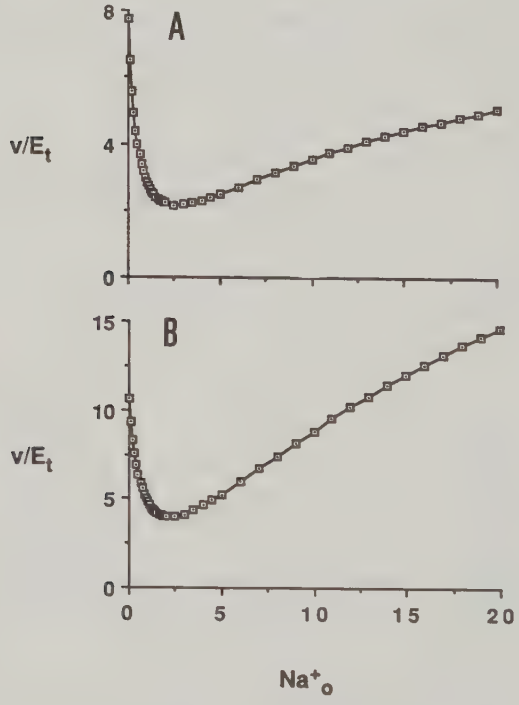

Figure 2. Effect of the external [Na^+] on the Na-ATPase (A) and the ATP/ADP exchange (B) activities in sealed preparations.

Fig. 2A illustrates a plot of Na-ATPase against Na_o^+ concentration in sealed preparations. This figure shows the initial high affinity inhibition of the Na-ATPase by external Na^+ and the following stimulation at higher cation concentration. A similar pattern was obtained for the ATP/ADP exchange velocity (Fig. 2B). Plots for the ATP/ADP exchange and ATP hydrolysis in broken membranes at different Na^+ concentrations are shown in Fig. 3. Both activities increase steeply as the [Na^+] is raised from 0 to about 2 mM,

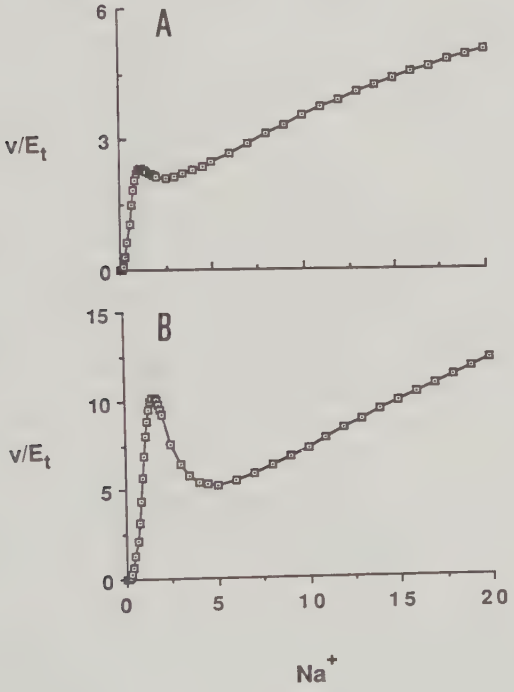

Figure 3. Effect of the [Na^+] on the Na-ATPase (A) and the ATP/ADP exchange (B) activities in broken membrane preparations.

slightly decrease between 2 and 5 mM and then continue to rise with increasing Na^+ levels.

Na^+ stimulates Na-ATPase and ATP/ADP exchange activities up to 150 mM in both sealed and broken membranes. At higher concentrations, Na^+ stimulates the ATP/ADP exchange but inhibits the Na-ATPase activity (not shown).

CONCLUSIONS

Computer simulation of a kinetic model similar to the Post-Albers scheme reproduce the experimentally observed high affinity Na^+ inhibition of the Na-ATPase and ATP/ADP exchange activities. The inhibition is mainly produced by a displacement in the steady-state concentration from the E_2P to the Na^+E_2P forms of the enzyme when Na^+ concentration increases between 0 and 5 mM. The inhibition is reversed by Na^+ binding to the K^+ sites and stimulating dephosphorylation. The interplay between the two sets of external Na^+ sites explains the experimental observation that Na-ATPase is inhibited at high [Na^+] but not the ATP/ADP exchange activity while both activities are simultaneously activated at low [Na^+]. The importance of this study resides not only in the explanation of some experimental observations but also in the demonstration that to understand the effects of external Na^+ it is not necessary to assume any more Na^+ sites than are implicit in the Post-Albers model.

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PROTON EFFECTS ON THE SODIUM PUMP

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INTRODUCTION

The Na,K-ATPase catalyzes several pump modes other than 'normal' Na^+/K^+ exchange associated with the forward operation of the entire pump cycle. These modes include ouabain-sensitive ATP-dependent Na^+ efflux from the cytosol into alkali cation-free extracellular medium and, in the absence of cytosolic Na^+, translocation of Rb^+ or, presumably, K^+, in the opposite direction. Both modes have been observed in experiments with human red cell Na,K-ATPase (for review see Hoffman, 1986). The former mode, referred to originally as uncoupled Na^+ efflux and designated here as the $Na^+/0$ mode is an electroneutral Na^+-anion cotransport, at least at physiological pH (Dissing and Hoffman, 1983). The latter mode is barely detectable at physiological pH and occurs mainly at acidic pH (Blostein, 1985); it may reflect pump mediated $H^+/K^+(Rb^+)$ exchange, particularly since Hara and co-workers observed an ATP-dependent ouabain-sensitive intravesicular acidification of K^+-filled kidney Na,K-ATPase proteoliposomes (Hara and Nakao, 1986).

In view of the foregoing behaviour as well as reports that ouabain-sensitive ATP hydrolysis in the absence of alkali cations is stimulated by a decrease in pH (Hara et al., 1986), we have examined further the possible role of protons as congeners not only of Na^+ at cytoplasmic site(s) as suggested previously (Blostein, 1985), but also K^+ at extracellular site(s).

RESULTS AND DISCUSSION

The experiments described in this paper were carried out with inside-out vesicles derived from human red cells, essentially as described earlier (Blostein, 1983 and 1985), K^+ being replaced by Rb^+. ATP-dependent $^{22}Na^+$ influx and $^{86}Rb^+$ efflux are equivalent to normal Na^+ efflux and Rb^+ influx in intact cells, respectively; as in earlier reports, the sidedness of the cations are designated by the subscripts "ext" and "cyt" to denote the extracellular and cytoplasmic milieu, respectively. The pH was varied, usually by titrating a constant amount of Tris (5 or 10 mM) with either MES or glycylglycine.

Protons as Na^+ Congeners

As shown in Fig. 1A and as described earlier (Blostein, 1985), ATP-dependent $^{86}Rb^+$ efflux is almost undetectable in the absence of Na_{cyt} at physiological pH and is increased markedly when the pH is decreased. This

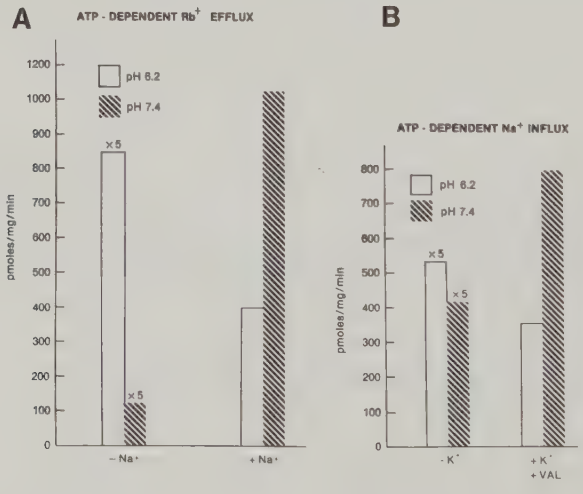

Figure 1. Effects of pH on pump mediated fluxes. A. Vesicles equilibrated with 0.3 mM $^{86}RbCl$ were assayed for 3 minutes with or without 2 mM NaCl; $[ATP] = 0.5$ mM. B. Vesicles equilibrated with 0.3 mM KCl were assayed for 4 minutes with or without 2 mM NaCl; $[ATP] = 0.1$ mM.

is in contrast to $^{86}Rb^+$ efflux via normal Na^+/Rb^+ exchange which is decreased as pH is decreased. In the representative experiment shown (Na_{cyt} present), the activity is reduced over 50% as pH is decreased from 7.4 to 6.2.

In the absence of Na_{cyt}, the increased Rb^+ flux caused by lowering the pH is consistent with a Na^+-like effect of H^+. Accordingly, it would be expected that, in the absence of Na^+, lowering the pH should enhance formation of K_{ext} (Rb_{ext})-sensitive phosphoenzyme (EP). In the experiment depicted in Figure 2, we measured the steady-state level of EP in the absence and presence of Na_{cyt} and tested the effects of Rb_{ext} at pH 7.4 and pH 6.2. As shown, Rb^+-sensitive EP is apparent in the absence of Na^+ only at acidic pH.

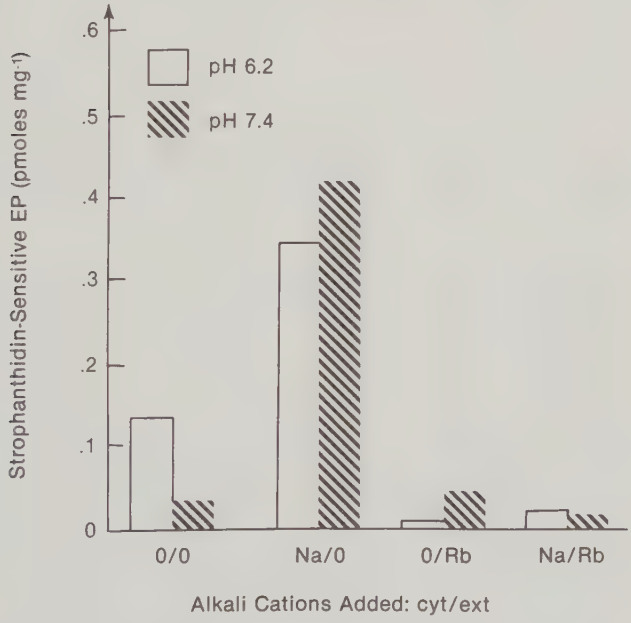

Figure 2. Effects of pH on EP. Vesicles equilibrated with or without 0.3 mM RbCl (Rb_{ext}) were assayed in media with or without 2 mM Na_{cyt} as indicated; [ATP] = 0.1 μM

If protons can, indeed, substitute for Na^+, we reasoned that the Na^+/ATP coupling ratio (ratio of Na^+ ions translocated per molecule ATP hydrolyzed) might be decreased as pH is decreased. To test this possibility, ATP-dependent $^{22}Na^+$ influx (normal efflux) and strophanthidin-sensitive ATP hydrolysis were measured concurrently under identical conditions (Harvey and Blostein, 1986). The results shown in Table 1 are consistent with Na^+ ions being replaced by protons: with vesicles carrying out normal Na^+/Rb^+ exchange, the Na^+/ATP ratio decreased from 1.7 at pH 7.6 to 0.8 at pH 6.2; in the absence of extracellular alkali cations (Na^+/0 mode), the ratio decreased from 1.6 to 0.8 . The relatively low ratio observed at pH 7.6 is due, presumably, to the hydrolytic activity of leaky or fragmented vesicles.

TABLE 1. Effects of Acidic pH on the Coupling Ratio

Exp.	Ratio	pH			Pump Mode
		6.2	7.6	8.0	
A	Na^+/ATP	0.75	1.55	-	Na^+/0
	Na^+/ATP	0.81	1.71	-	Na^+/Rb^+
	Rb^+/ATP	0.41	0.75	-	Na^+/Rb^+
	Na^+/Rb	1.95	2.22	-	Na^+/Rb^+
B	Rb^+/ATP	0.40	-	1.62	Na^+/Rb^+
C	Rb^+/ATP	0.40	-	-	0/Rb^+

Vesicles equilibrated without or with 0.3 mM RbCl were assayed in media containing 10μM ATP with or without 2 mM NaCl as described by Blostein (1983) and Harvey and Blostein (1986).

Protons as K^+ Congeners

The possibility that protons act as K^+ congeners was evidenced first in an unexpectedly low Rb^+/ATP

coupling ratio at pH 6.2. This was observed both in the absence of Na^+, consistent with H^+/Rb^+ exchange, as well as in the presence of Na^+ (Na^+/Rb^+ mode). Thus, at pH 6.2 the Rb^+/ATP ratio in both cases was 0.4 (Table 1). For the Na^+/Rb^+ mode the ratio increased to 1.6 as the pH was raised to 8.0.

Although the pump mediates ATP-dependent anion-coupled Na^+ efflux at physiological pH (Dissing and Hoffman, 1983) the question remains whether protons can replace K^+ and thus stimulate Na^+ efflux. We compared ATP-dependent $^{22}Na^+$ influx via normal Na^+/K^+ exchange and that observed in the absence of extracellular (intravesicular) alkali cations. As shown in Fig. 1B (right-side), normal Na^+/K^+ exchange is reduced over 50% (c.f. Fig. 1A) whereas the Na^+/O activity (left-side) is increased. If one assumes that the decrease in Na^+/K^+ exchange reflects overall reduction in catalytic function, the true stimulation of the Na^+/O flux is much greater than apparent.

Further evidence for a role of protons as K^+ congeners was obtained from measurements of strophanthidin-sensitive ATP hydrolysis. Whereas Na,K-ATPase activity is diminished markedly as pH is lowered, Na-ATPase activity is increased (Fig. 3). It is

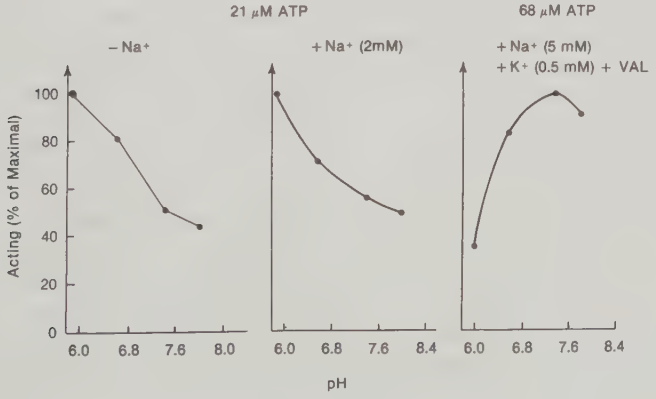

Figure 3. Effects of pH on strophanthidin-sensitive ATP hydrolysis.

important to note that stimulation of hydrolysis is also observed in the complete absence of alkali cations, consistent with the notion that protons are congeners of both Na_{cyt} and K_{ext}. In the absence of Na_{cyt}, however, little, if any, Rb_{ext} stimulation of hydrolysis is observed, even when the ATP concentration is raised to 100 μM. This raises the question whether the relative rates of step(s) in the reaction sequence are altered when the enzyme binds H^+ rather than Na^+.

With Na_{cyt} present, rudimentary evidence of a K_{ext}-like effect of extracellular (intravesicular) H^+ (H_{ext}) was obtained from measurements of the sidedness of pH effects on Na-ATPase as follows: Vesicles with markedly reduced anion permeability were prepared from extensively washed red cells which had been pretreated with 4,4'-diisothiocyano-2,2'-stilbene disulfonate (DIDS). In a typical experiment, vesicles were equilibrated at pH 6.2 or 7.5 and assays of ATP hydrolysis were carried out for 5 seconds at 37°C by diluting the vesicles 10-fold in media containing 2 mM NaCl at pH 7.8 or 7.5, respectively; the final extravesicular (cytoplasmic) pH was 7.5. Na-ATPase was increased when the initial intravesicular (extracellular) pH was decreased from 7.5 to 6.2, e.g. from 28.3 to 49.7 pmoles/mg/min, respectively. Since in these experiments the ATP concentration was low (0.1 μM), the observed ability of intravesicular (extracellular) protons to increase Na-ATPase suggests that, in contrast to K_{ext} or Rb_{ext}, but similar to Li_{ext}, deocclusion of protons is probably less rate-limiting at low ATP concentration (c.f. Beaugé and Del Campillo, 1976).

CONCLUSION

The notion that protons can act as Na^+ congeners as suggested in previous reports (Blostein, 1985; Hara and Nakao, 1986 and Hara et al., 1986) is substantiated further by the present experiments showing that K_{ext}-sensitive phosphoenzyme and strophanthidin-sensitive ATP hydrolysis are enhanced markedly in the absence of Na^+ when the proton concentration is increased. In addition, the reduction in the Na^+/ATP coupling ratio effected by reducing the pH provides indirect evidence that protons interact at site(s) involved in Na^+ translocation.

A role of protons as K^+ congeners is evidenced in the stimulatory effects of acidic pH on strophanthidin-sensitive ATPase, both in the absence and presence of Na_{cyt}, as well as on the rate of the Na^+/O flux. Reducing the pH reduces the Rb^+/ATP coupling ratio and thus provides indirect evidence for replacement of $K^+(Rb^+)$ by H^+. It is likely, however, that these effects of protons are distinct from those believed to effect shifts in the enzyme's conformation resulting in pH-dependent alterations in the enzyme's behaviour as described earlier by Skou and Esmann (1982).

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FUNCTIONAL CONSEQUENCES OF THE MEMBRANE POOL OF ATP ASSOCIATED WITH THE HUMAN RED BLOOD CELL Na/K PUMP

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It is becoming increasingly clear that the human red cell Na/K pump resides within the membrane as one component in a multienzyme complex. While the molecular organization of the complex and its structural arrangement in the membrane has yet to be defined, the functional consequence of some of the enzymes involved have now been established. Previous work (see Schrier, 1966) showed that the enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPD) and phosphoglyerate kinase (PGK) are not only membrane bound but can function to produce ATP (Schrier, 1967; Proverbio and Hoffman, 1977). It was also shown (Parker and Hoffman, 1967; Proverbio and Hoffman, 1977) that the ATP so produced was compartmentalized within the membrane forming a pool of ATP that could be preferentially used by the Na/K pump. A scheme depicting this enzyme complex is shown in Figure 1.

The basis for a membrane compartmentalized form of ATP comes from studies (see Proverbio and Hoffman, 1977) on porous ghosts concerned with factors that influence the formation of the pump's Na-phosphoprotein from bulk [γ-^{32}P]ATP, utilizing the methods as described by Blostein (1968). We found that when the membranes were preincubated with non-radioactive ATP and then thoroughly washed (to remove all excess ATP) prior to exposure to [γ-^{32}P]ATP

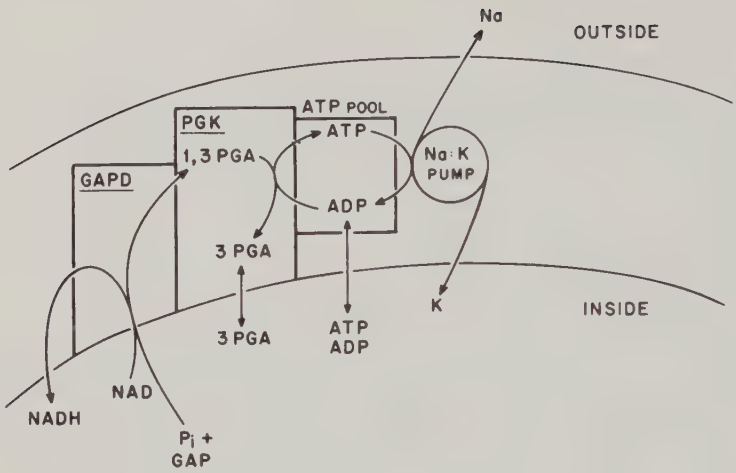

Figure 1. A scheme representing the membrane organization of GAPD and PGK in association with the Na/K pump. Addition of the substrates indicated (PGA is phosphoglycerate) generates ATP within the membrane pool that is preferentially utilized by the Na,K-ATPase. Substrates (including the adenine nucleotides) present in the cytoplasm (inside) have access to the membrane bound enzymes and ATP pool. (Figure from Proverbio and Hoffman, 1977).

there was no formation of ^{32}P-labelled Na-phosphoprotein by the pump even though formation of the phosphoprotein associated with the Mg-ATPase was unaffected. It turns out that the preincubation procedure filled a membrane pool with ATP and as long as that pool contained ATP, the formation of Na-phosphoprotein from bulk [γ - ^{32}P]ATP could not be seen (see Figure 2). Obviously labelling of the Na-phosphoprotein from bulk [γ - ^{32}P]ATP provides a probe for testing for the presence of pool ATP. By comparison with control membranes (not pretreated) it is possible to empty a pre-filled ATP pool in two ways prior to a test probe with [γ - ^{32}P]ATP: one way is to incubate membranes with Na+K, which uses up pool ATP by activation of the pump; the other way is to run the PGK reaction backward i.e. addition

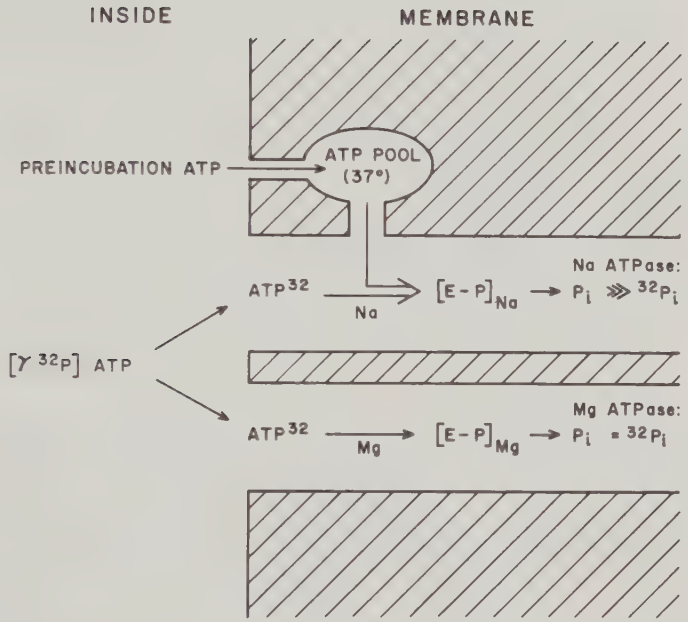

Figure 2. Interpretation of the use of pool ATP versus bulk (denoted as [γ - ^{32}P]ATP) by the Na,K-ATPase and Mg-ATPase. In the absence of pool ATP, bulk ATP can be used for the formation of the Na-phosphoprotein ([E-^{32}P]Na) and for the formation of $^{32}P_i$ liberated by the Na-ATPase. Both of these activities are inhibited by ouabain. When pool ATP is present (incorporated either by preincubation with ATP or by running the PGK reaction forward) no formation of [E-^{32}P]Na from bulk ATP is seen until the pool ATP is used up. In contrast, whether or not pool ATP is present, only bulk ATP is used by the Mg-ATPase for the formation of its phosphoprotein (E-^{32}P]Mg) and $^{32}P_i$ production. It may be that the only access of ATP to the Na/K pump is via the membrane pool, as indicated in Figure 1. (Figure from Proverbio and Hoffman, 1977.)

of 3-PGA and NADH converting ATP to ADP. For all the types of results described below both of these methods have been used to empty the pool of ATP, first filled either by preincubation with non-radioactive ATP or by running the PGK reaction forward. Mention should be made of an important study carried out by Mercer and Dunham (1981) who showed, using inside-out vesicles prepared from human red blood cells, that pool ATP alone can support the pumped transport of Na and K.

Given the above as background we now survey some of the functional consequences of pool ATP. Thus we try to determine whether pool ATP in contrast to bulk ATP can support certain known ATP-dependent processes.

Ouabain binding. It was known from previous work (cf. Hoffman, 1969; Bodemann and Hoffman, 1976) that bulk ATP markedly promotes the binding of ouabain to the ghost membrane and that this binding results in inhibition of the Na,K-ATPase. The present work established that pool ATP alone could also promote the binding of ouabain and that the ouabain so bound was inhibitory for the Na,K-ATPase.

Ouabain is known to bind to the human red cell membrane with very high affinity. While essentially no elution of ouabain (measured with [^{3}H]ouabain) occurs by incubation of membranes in the presence of high concentrations of K or non-radioactive ouabain, Huang and Askari (1975) found that previously bound ouabain is readily eluted by incubation of the membranes in the presence of Na and ATP, in the absence of Mg. In fact addition of Mg prevented the elution of ouabain. We found that ouabain bound to the membrane that was dependent upon and promoted solely by pool ATP (in the presence of Mg) could be eluted by the addition of EDTA to the medium. The elution of ouabain was dependent upon the presence of Na. In addition, the Na,K-ATPase activity was increased in proportion to the fractional release of bound ouabain. These results indicate that pool ATP can function to control the pump's interaction with ouabain.

p-nitrophenyl phosphatase (pNPPase). It was known from previous work (Rega, et al., 1968) that the K-sensitive and

ouabain inhibitable pNPPase activity of human red cell membranes is inhibited by Na and that this Na inhibition can be prevented by the addition of ATP to the medium. We found that pool ATP alone could also protect against Na inhibition but not after removal of ATP from the pool.

Caged-ATP. Caged-ATP is a protected form of ATP that upon photolysis with UV light liberates normal ATP (Kaplan, et al., 1978). We found that caged-ATP could be incorporated into the pool by the preincubation procedure and was ineffective in preventing the formation of the ^{32}P-labelled Na phosphoprotein until after photolysis. Photoreleased ATP could be shown to function the same as when the pool was loaded with ATP by preincubation.

Ca-ATPase (Ca_2 pump.) Pool ATP was found to prevent the formation of the ^{32}P-intermediate associated with the red cell Ca-ATPase (see Knauf, et al., 1974). The maximum inhibition in the formation of the Ca-dependent phosphointermediate seen was approximately 50% which may mean that there are more Ca pumps than pools of ATP in the membrane. Nevertheless, the fact that the Ca-ATPase is influenced by pool ATP indicates that the Ca pump is a component part of the multienzyme complex referred to earlier.

Location of the membrane ATP pool. It is estimated that there are approximately 500 molecules of ATP per membrane pool assuming one pool per Na/K pump. We do not as yet have substantive evidence identifying the membrane elements providing for and/or associated with the ATP pool. We have found that mild stripping of portions of the membrane cytoskeleton (using a modified procedure of Yu and Steck, 1975) eliminates the pool while leaving the Na,K-ATPase intact. Another approach involves the use of [α-^{32}P]8 azido ATP (Haley and Hoffman, 1974). [α-^{32}P]8 azido ATP can be put into the membrane pool via the PGK reaction using [α-^{32}P]8 azido ADP in place of ADP. [α-^{32}P]8 azido ATP (in the dark) acts like ordinary pool ATP but exposure to UV light inhibited the Na,K-ATPase and resulted in covalent labelling of membrane proteins. While we have been able to take the difference pattern (using SDS/PAGE)

of proteins labelled with the pool filled and emptied, the results are too preliminary to warrant reporting at this time.

Conclusion. It is clear from the foregoing survey of results that glycolytic enzymes as well as the Na/K and Ca pumps are associated in a membrane complex that involves a compartmentalized form of ATP. Indeed, the glycolytic rate of intact human red blood cells is sensitive to the rate at which the Na/K pump operates for as Whittam and Ager (1965) found ouabain-sensitive lactate production increased as the pump rate was increased by raising intracellular Na. While there is evidence accumulating that ATP is compartmented in a variety of cells (see for instance, Lipton and Robacker, 1983; Paul, 1983; Kurganov et al., 1985) the relation to the type of ATP pool described above for red cells has yet to be established.

Acknowledgments

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THE NA PUMP REACTION MECHANISM IS PING-PONG WITH RESPECT TO NA AND K.

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The discovery more than thirty years ago that the fluxes of Na and K through the Na pump are coupled led to the suggestion that the reaction mechanism is ping-pong (Cleland, 1963a) with respect to Na and K; that is, Na is released to the outside before K adds and K is released to the inside before Na adds. Shaw (1955) and Glynn (1956) proposed a mechanism which involved cyclic changes in the affinity of a carrier for Na and K; this mechanism forms the basis of the Albers-Post scheme. The proposal has been, and still is, the subject of debate.

Enzyme reaction mechanisms are evaluated by structural studies, binding studies, rapid reaction kinetic studies of individual steps, and steady state kinetic studies of the overall reaction mechanism. All four approaches are usually necessary, but may not be sufficient, to unambiguously demonstrate the validity of a given mechanism. In particular, steady-state methods provide a means for determining the sequence in which substrates add and products are released during the overall reaction. Since these methods make it possible to probe a reaction mechanism while the enzyme is carrying out its overall reaction, they allow one to be certain that partial reactions seen in the absence of one or more substrates, or under circumstances in which a single turnover is examined, are actually part of the overall reaction under turnover conditions rather than side reactions or alternate reaction pathways present when some substrates are missing and the complete reaction cannot take place. The evidence from steady-state studies that the Na pump is ping-pong with

respect to Na and K is now overwhelming, and I believe it would be useful to outline briefly the available data rather than recount the details of the poster which, at any rate, have been fully published (Sachs, 1986b).

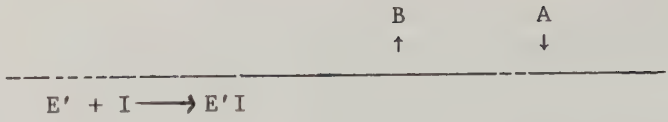
Steady-state methods for evaluating complex reaction mechanisms were worked out nearly twenty-five years ago (Segel, 1975). Bisubstrate reactions may be either ping-pong or sequential. Sequential mechanisms differ from ping pong mechanisms in that in a sequential mechanism both substrates (in the case of the Na pump, intracellular Na (Na_c) and extracellular K (K_o)) add to the enzyme before a product (extracellular Na (Na_o) or intracellular K (K_c)) is released. To distinguish between the mechanisms, it is necessary to measure the maximal velocity (V_M) and $K_{1/2}$ (concentration of substrate at which the velocity is half maximal) for one substrate at varying fixed concentrations of the other. It is important to note that such parameters are directly measured experimental values and make no assumptions about kinetic constants, dissociation constants or detailed mechanisms. For ping-pong mechanisms the ratio $V_M/K_{1/2}$ for one substrate does not vary with the fixed concentration of the other substrate, but for sequential mechanisms the ratio varies. Early studies of the Na pump found that the $K_{1/2}$ for Na_c or K_o did not vary with the fixed concentration of K_o or Na_c respectively (Hoffman and Tosteson, 1971; Garay and Garrahan, 1973), but the measurements were made in the presence of products (Na_o or K_c), and are therefore not readily interpretable. We repeated the experiments in K-free cells and Na-free solutions and found that $K_{1/2}$ for K_o as expected varied with the concentration of Na_c, but the ratio $V_M/K_{1/2}$ was not constant; it was a sigmoid function of [Na_c] (Sachs, 1977; Sachs, 1979). However, if we took account of the uncoupled Na efflux and included it in the ping-pong model, a rate equation resulted which exactly described the data. Moreover, using values for the relevant constants obtained by fitting the model to the experiments measuring Na-K exchange, the model predicted an uncoupled Na efflux in Na- and K- free solutions whose magnitude was just about what is found experimentally when Na efflux into solutions free of Na and K is measured. It perhaps need not be pointed out that this was not an ad hoc modification to fit the data, but rather a more accurate model which took into account the experimentally demonstrated existence of an uncoupled Na efflux (Glynn and Karlish, 1976). The simple

model which emerged from the data was

in which the curved arrow represents the uncoupled Na efflux; this is the basic model tested in the studies referred to below. It should be pointed out that a sequential mechanism without an uncoupled Na efflux could also describe these findings, but no sequential model with an uncoupled efflux has been proposed to account for the observations.

The presence of Na-Na and K-K exchanges carried out by the pump strongly support the ping-pong model provided they are part of the overall Na-K exchange and not side reactions. If, as in the sequential model, Na_c must add before K is released, the K-K exchange should require Na_c; in fact, it is inhibited by Na_c. Similarly, if K_o must add before Na is released, the Na-Na exchange should require K_o; in fact, it is inhibited by K_o. We recently reported experiments (Sachs, 1986a) in which we examined inhibition of the K-K exchange by Na_c; the results agreed in detail with predictions resulting from the hypothesis that the K-K exchange really is part of the overall Na-K exchange. Furthermore, in the course of these experiments we found that the rate equation derived from a ping-pong model which includes an uncoupled Na efflux (1) accounted for a previously puzzling finding that the $K_{1/2}$ for K_o of the Na-K exchange differs from the $K_{1/2}$ for K_o of the K-K exchange when the parameters were simultaneously measured in the same cells and (2) predicted the existence of a previously undiscovered uncoupled K efflux which we then demonstrated experimentally.

A third, and independent, approach to the determination of the pump reaction mechanism depends on the evaluation of the characteristics of inhibition by reversible inhibitors which combine with specific enzyme forms. The rationale for the method is intuitively obvious (Cleland, 1963b). If in the reaction scheme



an inhibitor combines with E′, the nature of the resulting inhibition depends on whether or not the steps between E′ and the point at which substrate A adds are reversible. The steps will be reversible if product B is present, and irreversible if it is absent. If the steps are reversible, E′ can be formed in the forward direction after addition of A, or in the reverse direction by addition of B. The steady-state concentration of E′ and therefore fractional inhibition by I will not vary with the concentration of substrate A. If the velocity is measured at varying substrate concentrations, the inhibitor decreases V_M but does not alter $K_{1/2}$; inhibition is noncompetitive with A. On the other hand if steps between E′ and the point at which A adds are irreversible, E′ is formed only in the forward direction after addition of A, and therefore the steady-state level of E′ and the fractional inhibition by I will increase with A. In this case, the inhibitor reduces both V_M and $K_{1/2}$ of plots of velocity versus substrate concentration; inhibition is uncompetitive with A. If, therefore, removal of a product converts noncompetitive inhibition with respect to a substrate to uncompetitive inhibition, release of the product must occur between the point at which the inhibitor adds and the point at which the substrate adds.

We have used this approach to confirm the order in which Na is released and K adds at the outside using oligomycin (Sachs, 1980), and to evaluate the order in which K is released and Na adds at the inside using vanadate (Sachs, 1986b). Oligomycin, which inhibits by combining with E_1 forms which have bound Na, is a noncompetitive inhibitor with respect to K_o in Na- free solutions. Similarly, vanadate, which inhibits by combining with E_2K, is a noncompetitive inhibitor with respect to Na_c when K_c is present, but an uncompetitive inhibitor in K-free cells. These results clearly mean that Na is released to the outside before K adds, and K is released to the inside before Na adds; i.e., the mechanism is ping-pong.

Several points can be made about these studies:

1) Steady state methods have been misused by considering only a single aspect of a complicated mechanism, and more than one reaction mechanism may account for an isolated observation. However, as the number and complexity of the experimental observations which must be explained by a single mechanism increases, the number of mechanisms which describe the data decreases, and a unique mechanism emerges. Scheme 1, without modification, described in detail the overall steady-state kinetics of Na-K exchange, the detailed kinetics of K-K exchange, the characteristics of inhibition by two reversible inhibitors, and predicts two experimentally verified uncoupled effluxes. To be taken seriously, any competing model must be shown to be as effective.

2) Since steady-state fluxes were measured, all of the studies described above examined properties of the transport sites under turnover conditions. The studies necessarily apply to circumstances in which Na_o and K_c are absent, and phosphate was usually low. It is possible that when these products are present alternative reaction pathways may appear. However, when the products are absent, the pump rate is maximal so that scheme 1 describes the pump reaction mechanism when the pump functions at maximal efficiency. If there are alternative pathways, they are modifications of the basic ping-pong mechanism.

3) In addition to the possibility of alternative pathways in the presence of Na_o and K_c, it is possible that these ions may modify the exchange cycle by interacting with sites other than the transport sites. Such interactions, if they exist, are not essential features of the reaction mechanism.

4) Single turnover experiments have demonstrated release of Na from the Na bound enzyme in the absence of K (Forbush, 1984) and release of K from the K bound enzyme in the absence of Na (Glynn and Richards, 1982) rapid enough to be part of the overall ping-pong reaction mechanism.

The original suggestion that the Na pump reaction mechanism is ping-pong with respect to Na and K now seems firmly established.

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HOW THE ENERGY FROM ATP HYDROLYSIS IS USED TO RENDER ION PUMPING EFFECTIVE

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Does pump asymmetry drive transport ?

We all know (or think we know) that an ion pump works by loading its substrate at a high affinity site at the side from which pumping occurs, and discharging the substrate from a low affinity site at the other face of the membrane. We feel that the energy from hydrolysis of ATP is used to bring about this change of the site affinities, and that this is what drives pumping. But Hill and Eisenberg (1981) showed us that this cannot be the whole story or even the major part of it. They showed that the release of the ATP's free energy can occur anywhere around the pumping cycle and yet pumping will still occur. Consider the conventional scheme for the Na/K ATPase (Fig 1):

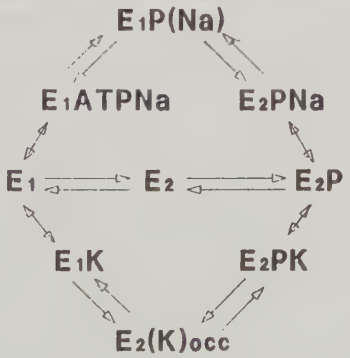

Figure 1

The upper half of the scheme show the pumping of sodium linked to the phosphorylation of the enzyme, the lower half, potassium pumping linked to dephosphorylation. Nowhere in the scheme as written is there any indication of a change of pump affiniy for either ion. Yet the scheme will pump sodium and potassium in opposing directions against their respective concentration gradients, if the splitting of ATP is tightly coupled to the ion movements. It is this tight coupling, Hill and Eisenberg emphasise, that drives the pump. A little reflection will convince one that pumping will occur, in a strictly coupled pump, even if the pump's affinities for ions are quite contrary to those ordinarily found. A pump can pump an ion which binds to it at a low affinity site at the side from which pumping is occurring (the "whence" side in what follows), and discharge it from a high affinity site at the side to which pumping is occurring (the "whither" side), provided only that the cycle of fig 1 continues to take place, that is, provided that the overall equilibrium between ATP, ADP and Pi, and the prevailing ion gradients are such as to allow ATP splitting to continue. It is thermodynamics that determines whether or not pumping will occur, not kinetics.

The simple carrier can be asymmetric and yet cannot pump.

Indeed, one can easily demonstrate the falsity of the assumption that an asymmetry of affinities must bring about pumping. We now know that many simple carrier systems, which cannot bring about the active transport of their substrates, are asymmetric in their kinetics as well as in their structure. Two well-studied examples are the glucose transport system and the uridine transport system of the human red blood cell (Baker and Widdas, 1973; Cabantchik and Ginsburg, 1977). Interestingly, and revealingly, both these systems develop their asymmetry on cold-storage of the cells, and they do this without developing any capacity for pumping their substrates (Weiser et al, 1983; Plagemann and Wohlhueter,1984). There is good evidence that it is the accumulation within the cells of some competitive inhibitor of transport that induces these asymmetries. Consider what will happen if a non-penetrating competitive inhibitor of transport is caused to be present at the cytoplasmic face of a carrier system. Within the cell, substrate and the inhibitor will compete. The effect on the efflux of the substrate is to

raise the effective Km, leaving Vmax unchanged. For influx, on the other hand, substrate and inhibitor do not compete - they are on opposite sides of the membrane. The Km for influx is unchanged and the system becomes asymmetric in its kinetics - the original high affinity at the extracellular face, but a low affinity at the cytoplasmic surface. Vmax for influx is, however, reduced. Even at the highest concentrations of substrate, the effect of inhibition at the cytoplasmic face cannot be overcome. Some of the carrier is trapped at the cytoplasmic face by combination with the competitor, reducing the amount of carrier available for transport of the substrate and hence reducing the Vmax. Indeed, the ratio of Vmax to Km must be the same for efflux as it is for influx, and this is achieved by raising Km for efflux, while lowering Vmax for influx.

The kinetic asymmetry of a carrier system depends on the difference in free energy between the carrier conformations that take part in transport. It is easy to show (Stein, 1986) that, for a carrier in which the loaded forms reorient across the membrane faster than do the unloaded forms (and this is true for the majority of the carrier systems), the asymmetry depends on the difference in free energy between the two conformations of the unloaded carrier. This is exactly true for the important exchange-only carriers. Since for all substrates that ride on the same carrier, the unloaded forms are the same, the kinetic asymmetry for all such substrates is identical, a strong result. This means that whatever are the intrinsic affinities for carrier and substrate at any face of the membrane, the ratio of affinities that a steady-state kinetic experiment will measure will be determined only by the free carrier's free energy differences. (In the case of a carrier plus competitive inhibitor system, the free energy difference takes into account the free energy change for the binding of competitive inhibitor to the carrier at the prevailing concentration of the inhibitor, as well as the free energy difference between the unloaded forms of the carrier.) Later we shall use an important corollary of this result : If a carrier system behaves symmetrically in its kinetics, the two forms of its unloaded carrier do not differ in free energy i.e. they are iso-energetic.

Pump asymmetry renders a pump effective.

What does all this have to do with ion pumping? We have just shown that asymmetry does not in itself lead to pumping. Yet we know that ion pumps are asymmetric. Is this fact of significance for pumping? The answer is that asymmetry increases the effectiveness of an ion pump. While it it true that a pump will pump with ion affinities low at the whence side and high at the whither, it will operate very slowly under these conditions. Much of the pump enzyme will be trapped unloaded at the whence side, unable to take part in the pumping process. Much of the time the pump will return loaded with its substrate from the whither side, having shuttled substrate fruitlessly across the membrane. (Both these situations waste pumping time not pumping energy (Honig and Stein, 1978). An ion that returns along the path from the whither to the whence side will resynthesise ATP by reversal of the splitting process. No energy is lost, only time.)

Time is of significance since our ion pumps are not the isolated systems depicted in fig 1. They are embedded in natural membranes which are leaky to the pumped substrates. At the steady-state, the pumped substrate will leak back down the built-up concentration gradient just as fast as pumping occurs. This wastes the energy that was invested into ion pumping. A pump will be effective if it is designed so as to have that high affinity at the whence side and low affinity at the whither side that we have always known was important for pumping. We now see that this aspect of pump design is required to render ion pumping effective by speeding up the pump cycle, a kinetic problem not a thermodynamic one, and neglected in the Hill-Eisenberg analysis.

How asymmetry is maintained in the potassium half of the pump cycle.

Consider, first, the simpler, potassium half of the pump cycle. We have shown (Karlish and Stein, 1982 a,b) that, in the absence of sodium and hence of ATP splitting, the system behaves as a simple carrier for potassium ions. Net transport is slower than exchange transport so that the unloaded carrier reorients more slowly than does the potassium - loaded form. In the absence of ATP and Pi, the system behaves symmetrically, kinetically, with high affinity sites for potassium being demonstrable at both

faces of the membrane. This must mean (Stein, 1986) that the two carrier conformations E1 and E2 must be more-or-less isoenergetic. But the intrinsic affinities of E1 and E2 for potassium are very different, being high at the extracellular face and low at the cytoplasmic face, by about two orders of magnitude. These affinities are just in the ratio that effective pump design would require (high at the whence side for potassium - the extracellular face; low at the whither side - the cytoplasmic face). How are these intrinsic affinities realised during potassium pumping? It is clear that ATP binds to E2 forms with a low affinity, to E1 forms with a high affinity. Hence the addition of ATP stabilises the E1 form, allowing its low affinity for potassium to be realised. We have the operation of the thermodynamic "box" depicted in fig 2,a. Whether one travels from E2 + ATP + K to E1 + ATP + K by first binding ATP and then K, or K first and then ATP, the initial and final states are the same, so the free energy difference is independent of route. Thus, if ATP binds poorly to E2 and well to E1, K must in like but reverse ratio, bind poorly to E1 and well to E2. Essentially, ATP acts as a competitive inhibitor of potassium movement, its action being localised at the cytoplasmic face, just as if it was one of the non-penetrating competitive inhibitors that we discussed above. This role for ATP is merely a regulatory one, and it is not split in the process. (If ATP is not split, the change of affinity - important for effective pumping - is not what brings about pumping.) The asymmetry in binding of potassium is given by the free energy difference for the binding of ATP to the two forms E1 and E2 of the potassium carrier (here the Na/K ATPase).

The forms E2 and E2P are not very different in free energy, the phosphate bond here being a low energy bond. The effective affinity of K for the system at side 2 is much the same whether or not Pi is present. Thus the asymmetry of potassium binding at its whence and whither sides does not depend on the difference of free energy between E1 and the phosphate form E2P, but rather on the differential binding of ATP to these two forms.

How asymmetry is maintained in the sodium half of the pump cycle.

The cytoplasmic face of the pump is the whence side

for

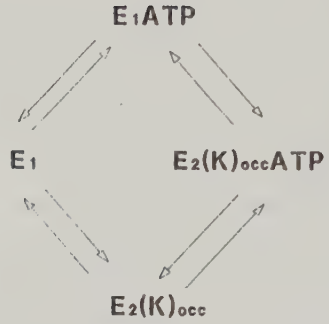

Figure 2

sodium but the whither side for potassium. Thus we cannot use ATP binding to ensure that both sodium and potassium bind more avidly at their whence faces. The differential binding of ATP to the E1 form means a lower affinity for ALL substrates to this form. Some other design principle is needed to generate a high affinity for sodium at the cytoplasmic face. The solution taken is a kinetic one. The complex Na.ATP.E1 takes a different route to E2 than does the complex K.ATP.E1, the ion which is bound determining the fate of the E1+ATP complex. Ion binding is the fate-determining step!

K.ATP.E1 merely undergoes a conformation change to yield the occluded form ATP.E2(K). In contrast, Na.ATP.E1 undergoes, of course, a chemical change with eventual phosphorylation of the enzyme and its reorientation to yield Na.E2.Pi + ADP. The reaction of phosphorylation followed by reorientation is heavily over to the side of Na.E2.Pi, so that the E2 form is stabilised over the E1 form, or E1 destabilised over E2. The binding of Na to E1 is thus followed kinetically by a change which removes the Na.E1 complex as fast as it is formed. Thus the binding of sodium to the carrier is heavily stabilised and the system behaves with a high affinity for sodium. Since, as we have seen, E1 and E2P are more-or-less isoenergetic, ALL of the chemical energy in splitting ATP to ADP + (Pi bound to E2) can be used to stabilise this binding of sodium to the pump from side 1, and sodium can bind with the required high affinity at its whence side. Splitting of ATP drives the whole pump cycle thermodynamically, but effective pump design ensures that the splitting of ATP takes place in a particular step

which ensures the satisfaction of the kinetic requirement for a high affinity for sodium at its whence side.

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Comparison of Na^+, K^+-ATPase, H^+, K^+-ATPase, and Ca^{2+}-ATPase

The Na^+,K^+-Pump, Part A: Molecular Aspects, pages 585-602
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OVERVIEW:
H,K-ATPase : Na,K-ATPase's STEPSISTER

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INTRODUCTION

In gastric acid secretion a large proton gradient is formed between blood and the gastric lumen. The proton concentration in the stomach is at least six orders of magnitude larger than in in blood. For the formation of such a gradient much energy is needed. The idea that an ATPase could be involved in such a process has been investigated for a rather long period. Earlier studies towards a direct role of Na,K-ATPase were negative (Hansen et al, 1975), although the potassium gradient formed by this enzyme was shown to be important for the acid secretion process. Forte and his coworkers (1967) showed the presence of a potassium-stimulated p-nitrophenylphosphatase activity in pig gastric mucosa and later they established that this activity was due to a potassium-stimulated ATPase, which could not be inhibited by ouabain (Ganser and Forte 1973). Lee, Simpson and Scholes (1974) carried out a crucial study in this field. They prepared a membrane fraction from pig gastric mucosa and added ATP to the incubation medium. Alkalinization occurred, apparently due to the uptake of protons by the membrane fraction. No alkalinization was found in the presence of detergents or ionophores, indicating that the preparation consisted of closed vesicles. Later studies by Sachs et al (1976) showed that the ATPase catalysed an electroneutral proton / potassium exchange and the enzyme was called H,K-ATPase by most investigators in this field. The enzyme has been shown to be present in the tubulovesicular system and in the apical membrane of the mammalian parietal cell. The enzyme is absent in other tissues with the possible exception of rabbit colon where recently a similar activity has been demonstrated (Kaunitz and Sachs, 1986).

The vesicles containing H,K-ATPase originate from the tubulovesicular membranes, and are mostly obtained from unstimulated gastric mucosa. Isolation of the enzyme in its pure form from stimulated tissue is more difficult and the

enzyme is then found in membranes with a higher density (Wolosin and Forte, 1981). These membranes also contain actin (Wolosin, 1985). Upon stimulation translocation of the enzyme occurs from the tubulovesicular system towards the apical membrane, either by fusion of these vesicles with this membrane (Forte et al, 1977) or by an unfolding of a preexisting structure (Berglindh et al, 1980 a).

TRANSPORT STUDIES

One of the discussion points regarding this enzyme is the ratio between ATP hydrolysis and ions transported. Values of 1 (Reenstra and Forte, 1981; Smith and Scholes, 1982), 2 (Rabon et al, 1982 a) and even 4 (Sachs et al, 1976) have been reported. A study using isolated vesicles from pig gastric mucosa yielded a variable ratio (Skrabanja et al, 1984). At infinitely high ATP concentrations the ratio between protons transported and ATP hydrolysed reached a value of nearly 2. Moreover the Hill coeficient of proton transport was significantly higher than 1, suggesting that two protons are transported together at the expense of hydrolysis of one molecule of ATP.

Isolated gastric vesicles however have the disadvantage that in addition to H,K-ATPase many other proteins are present. This could in principle influence the measurement of this ratio. We therefore decided to purify the enzyme in order to remove these contaminating proteins. Upon purification these proteins can indeed be removed, but this also leads to an increased leakiness of the vesicles, so that proton uptake studies are no longer possible.

In collaboration with A. Soumarmon and M. Lewin (Paris) purified H,K-ATPase has therefore been reconstituted in phophatidylcholine / cholesterol liposomes by means of a freeze-thaw / sonication technique (Rabon et al, 1985). The proteoliposomes were separated from non-incorporated enzyme and again the initial rates of ATP hydrolysis and proton uptake and additionally the rate of 86Rb-extrusion were determined (Skrabanja et al, 1987). The ratio between the maximal rate of either proton transport or 86Rb transport and of ATP hydrolysis approached again the value of 2, confirming the observations with the isolated vesicles. The maximal rates of proton and 86Rb transport were not significantly different confirming the electroneutrality of H,K-ATPase.

The studies with the reconstituted enzyme (Skrabanja et al, 1986) yielded other interesting information. When a potassium gradient from inside to outside was present the proteoliposomes took up protons in the absence of ATP (Fig. 1). The rate of this passive proton/potassium exchange was approximately 5% of that of the ATP-stimulated exchange. This passive transport could be inhibited by vanadate and by omeprazole, a specific H,K-ATPase inhibitor.

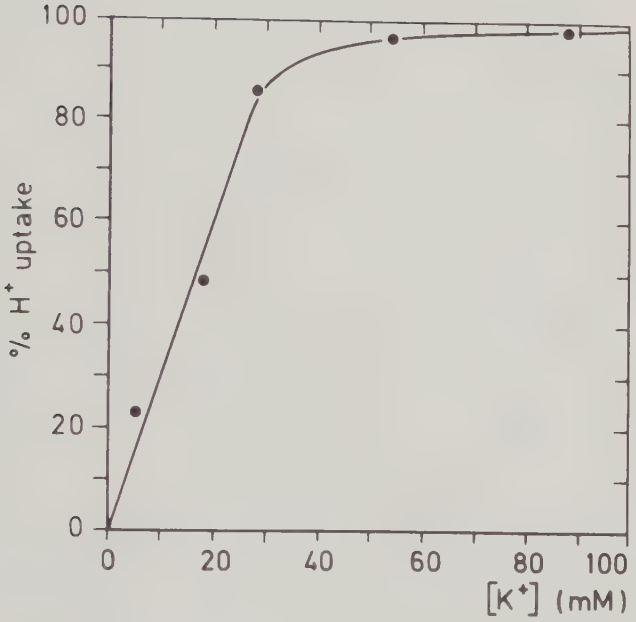

Figure 1. Passive hydrogen transport by reconstituted H,K-ATPase. The intravesicular potassium concentration is indicated on the x-axis. The proteolipomes were 6.7 times diluted in a potassium-free medium. From Skrabanja et al, 1986.

These proteoliposomes were also able to catalyse a passive potassium/potassium exchange with a rate which is nearly equal to that of the ATP-activated proton/potassium transport (Soumarmon et al, 1984, Rabon et al, 1985). This is in contrast to studies on Na,K-ATPase in which the rate of passive potassium/potassium exchange is much lower than that of ATP-stimulated sodium/potassium exchange (Karlish et al, 1982). Since in both enzymes the potassium/potassium exchange is assumed to represent the E2-E1 conversion, this step cannot be rate limiting in H,K-ATPase.

STRUCTURAL STUDIES

Purification of H,K-ATPase by gradient centrifugation followed by free flow or zonal electrophoresis, leads to

accumulation of a 100 kDa band in SDS gel electrophoresis. The mobility of this band is equal to that of the α-subunit of Na,K-ATPase. We could not find any immunological cross reactivity between the main subunits of Na,K-ATPase and H,K-ATPase (Peters et al, 1984), but Lane et al (1986) found that antibodies raised against the α-subunit of lamb kidney Na,K-ATPase cross reacted with the 100 kDa subunit of pig gastric H,K-ATPase.

There is still no final answer in the debate whether the 100 kDa subunit in H,K-ATPase is homogeneous or not. Upon isoelectrofocussing the 100 kDa band is split in several bands, but this can be due to heterogeneity in sugar composition. Smolka et al (1983) could elicit monoclonal antibodies against several of these bands. Saccomani et al (1979) carried out a tryptic digestion study on H,K-ATPase and concluded that the 100 kDa band consisted out of three different proteins. This conclusion was based on the fact that 34% of the 100 kDa subunit remained undigested and also from the observation that the 78 kDa band obtained after tryptic digestion in the presence of ATP could not be phosphorylated and appeared therefore to originate from a different protein than the catalytic subunit.

This study was repeated and extended by Helmich-de Jong et al (1987). They found a linear correlation between the amount of 100 kDa subunit left and the residual H,K-ATPase activity. Only 8% of the protein remained undigested. The 100 kDa band was split in bands with apparent molecular masses of 55 and 42 kDa in the case that potassium was present in the digestion medium. With ATP (and no potassium) present the digestion process was slower and bands were formed with apparent molecular masses of 67 and 35 kDa. The 42 and 67 kDa bands could be phosphorylated in contrast to the 55 and 35 kDa bands (Fig.2). These findings take away the basis for the conclusion on heterogeneity put forward by Saccomani et al (1979). Peters et al (1982) also challenged the conclusion of heterogeneity. Sedimentation studies did not reveal heterogeneity. No different N-terminal amino acids were detected. Alkylation with citraconic anhydride gave a clear shift of the 100 kDa band on SDS polyacrylamide gel without band splitting or widening and concanavalin A binding showed that 86% is a glycoprotein. Shull and Lingrel (1986) recently deducted the amino acid sequence of the 100 kDa subunit of H,K-ATPase by determining the nucleotide sequence of cDNA from rat gastric mucosa. This chain consisted of 1033 amino acids, accounting for a molecular mass of 114,012 Da. There was a striking homology between the amino acid sequence and the hydropathy plot of rat gastric H,K-ATPase and sheep kidney Na,K-ATPase.

The overall homology was 62%. The greatest homology was found around the phosphorylation site, in the region between the transmembrane fragments 2 and 3 and in the sequence preceding the transmembrane fragment 5, which may be part of

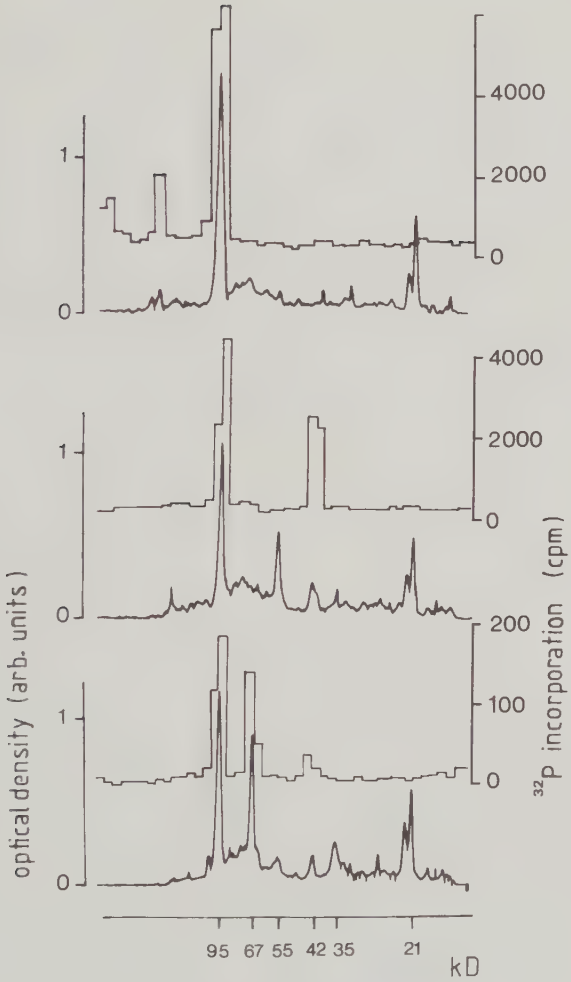

Figure 2. Phosphorylation capacity of tryptic peptides of H,K-ATPase. H,K-ATPase was subjected to tryptic digestion in the presence of either 100 mM potassium (middle panel) or 5 mM ATP, 1 mM EDTA plus 100 mM choline chloride (lower panel) for 5 and 30 min respectively. Protein pattern in SDS-gelelectrophoresis and 32P-counts after phosphorylation with ATP were compared. Top panel shows a H,K-ATPase preparation not treated with trypsin. From Helmich-de Jong et al, 1987.

the ATP-binding domain (Ohta et al, 1986). An interesting finding in this study is the fact that in the 5' region of the messenger RNA there is a reading frame encoding for a second polypeptide. This polypeptide containing 40 amino acids has a potential N-glycosylation site. Whether this peptide is indeed synthesized and whether it is a functional protein has to be established.

The discussion on homogeneity of the 100 kDa subunit of H,K-ATPase continues because of the stoichiometry of the phosphorylation level. With ATP the maximal phosphorylation level obtained is 1.6 nmole/mg protein (Faller et al, 1983), which is 1 mole per 650,000 g protein. Even when one takes impurities in the enzyme preparation and inaccuracies with the protein assay into account, one never reaches the level that each 100 kDa subunit can become phosphorylated. With other substrate probes approximately two times higher binding or phosphorylation levels (in nmole/mg protein) have been obtained: with inorganic phosphate: 2.5-2.7 (Jackson and Saccomani, 1983; Helmich-de Jong et al, 1986 b) with adenylyl imididiphosphate: 2.8 (Van de Ven et al, 1981), with azido-ATP (Faller et al, 1982) and with vanadate (Faller et al, 1983): 3.0, and both with eosin (Helmich-de Jong et al, 1986 a) and with fluorescein 5'-isothiocyanate (Farley and Faller, 1985): 3.5. With azido-ATP as well as with vanadate there is a suggestion that two different sites are involved. These findings can be interpreted by the existence of two binding sites present in equimolar amounts, one leading to an acid-stable phosphorylated intermediate, the other only able to bind some substrate analogues, but not able to yield an acid-stable intermediate. Rabon et al (1986) obtained crystalline arrays of gastric H,K-ATPase in a medium containing potassium, vanadate, magnesium and dimethylsulfoxide, which could accomodate 2 polypeptides of mass 116-129 kDa. Radiation inactivation studies suggest molecular masses of 270 kDa (Saccomani et al, 1981) or 371-444 kDa (Schrijen et al, 1983). The lower value was obtained in a frozen preparation, but the obtained value was not corrected for the temperature effect, which may have resulted in an underestimation of the molecular mass. Soumarmon et al (1983, 1986) determined in an active octylglucoside solubilized enzyme preparation approximate molecular masses of 390-420 kDa by gel filtration and glycerol gradient centrifugation. They also found that depolymerisation occurred leading to inactivation of the enzyme activity and smaller molecular masses. These studies and the radiation inactivation studies of Schrijen et al (1983) which were obtained with a lyophilized preparation suggest that the active enzyme is a tetramer. Other interpretations of these findings can, however not be excluded.

MECHANISTIC STUDIES

Like in Na,K-ATPase a large number of studies has been carried out in order to explain the coupling between ATP hydrolysis and ion transport. It is impossible to give in this section a comprehensive review, but the autors restrict themselves to some recent findings from their own work. In general the Albers-Post scheme is also applicable to H,K-ATPase, with the difference that protons take over the place of sodium in Na,K-ATPase. However, since protons are always present in an aqueous solution they cannot like sodium be omitted from the medium. This makes interpretation of results more difficult than with Na,K-ATPase. H,K-ATPase can, like Na,K-ATPase be phosphorylated at an aspartyl residue (Walderhaug et al, 1985). The rate of phosphorylation is increased by lowering the pH at the extracellular side (Stewart et al, 1981). The rate constants

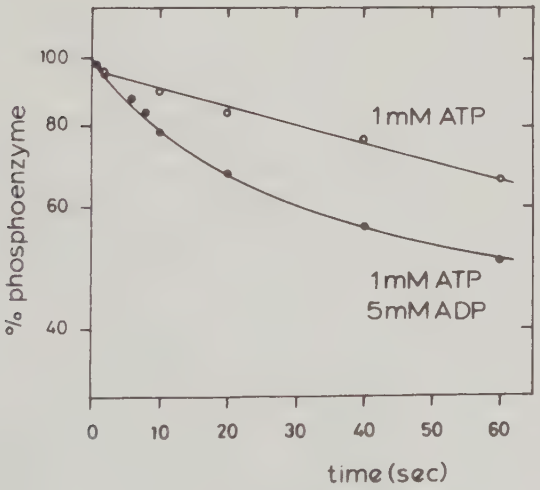

Figure 3. Effects of ADP and ATP on the dephosphorylation rate of the phosphorylated intermediate of H,K-ATPase obtained with ATP. Phosphorylation was carried out for 10 s at room temperature. Dephosphorylation was started by addition of non-radioactive ATP and stopped by adding trichloroacetic acid plus inorganic phosphate. From Helmich-de Jong et al, 1985 .

of phosphorylation are sufficiently high to allow the phosphoenzyme to be an intermediate in the overall reaction (Ljungström et al, 1984). The rate of dephosphorylation can be enhanced by potassium, but until recently no stimulatory effect of ADP on the dephosphorylation process was observed. An ATP/ADP exchange reaction was however shown to be present (Rabon et al, 1982, Schrijen, 1981).

Helmich-de Jong et al (1985) found recently a stimulatory effect of ADP on the rate of dephosphorylation. They phosphorylated H,K-ATPase at very low magnesium concentration and stopped the phosphorylation reaction by diluting the 32γ-ATP with 1 mM non-radioactive ATP (Fig.3). They found that 20% of the phosphorylated intermediate was sensitive to ADP. When however, the phosphorylation reaction was carried out with higher magnesium concentration or when the phosphorylation was stopped by complexing magnesium with EDTA, no stimulation of the dephosphorylation process by ADP was found.

This apparent discrepancy led to a further study (Helmich-de Jong et al, 1986 b). Comparison of the obtained results indicated that the rate of unstimulated dephosphorylation kinetically depended on the method used for stopping phosphorylation. The rate of dephosphorylation was much smaller when the phosphorylation process was stopped by diluting the radioactive ATP concentration, than by complexing magnesium with EDTA. Addition of ATP to EDTA again lowered the rate of dephosphorylation up to the rate with ATP alone. The 50% stimulatory concentration for this ATP effect was 0.1 mM, indicating that it occurs through a low-affinity binding site. Addition of magnesium to ATP increased again the rate of dephosphorylation (Fig.4). Interpretation of these complex findings was not simple. The effect of ATP could be explained by a general inhibitory effect of nucleotides on the dephosphorylation rate. This explains why Helmich- de Jong et al (1985) found a stimulatory effect of ADP on the dephosphorylation rate and others did not. When there is no ATP in the dephosphorylation medium, there is both a stimulatory and an inhibitory effect of ADP on the dephosphorylation rate, which effects may compensate each other. With ATP in the dephosphorylation medium there is only a stimulatory effect of ADP, since the inhibitory effect occurs through ATP and is already maximal. Three possibilities have been postulated to explain the stimulatory effect of magnesium on the dephosphorylation rate in the presence of ATP. First magnesium could complex ATP and change the free ATP or the MgATP concentration. Experiments in which various magnesium concentrations were used, showed this not to be the case. A second possibility would be that magnesium like potassium has a stimulatory effect on the dephosphorylation rate independent of ATP. This also was found not to be true.

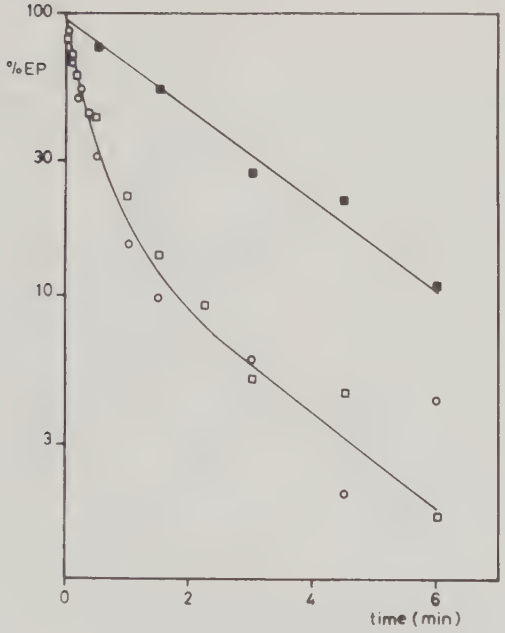

Figure 4. Effects of EDTA, ATP and magnesium on the dephosphorylation rate of the phosphorylated intermediate formed from ATP. Phosphorylation was carried out for 10 s at room temperature. Dephosphorylation was initiated by adding 1mM EDTA (open circles), 1mM ATP (closed squares) or 1 mM ATP plus 5 mM magnesium (open squares). Dephosphorylation was stopped as in Fig.3. From Helmich-de Jong et al, 1986 b.

Magnesium had no stimulatory effect on the dephosphorylation rate, when the phosphorylation reaction was stopped by diluting the radioactive ATP with cold ATP, leading to ATP concentrations, high enough to lower the radioactive ATP level, but not high enough to inhibit the dephosphorylation rate. Moreover magnesium had in contrast to potassium no stimulatory effect on the dephosphorylation rate of the phosphorylated intermediate made from inorganic phosphate. The third explanation, a lowering by magnesium of the affinity of nucleotides for its binding site seems the most likely one. A competition between ATP and magnesium on the rate of dephosphorylation could be demonstrated (Helmich de Jong et al, 1986 b).

These experiments indicate the presence of a low-affinity nucleotide binding site on H,K-ATPase, which can be found on the phosphorylated enzyme.

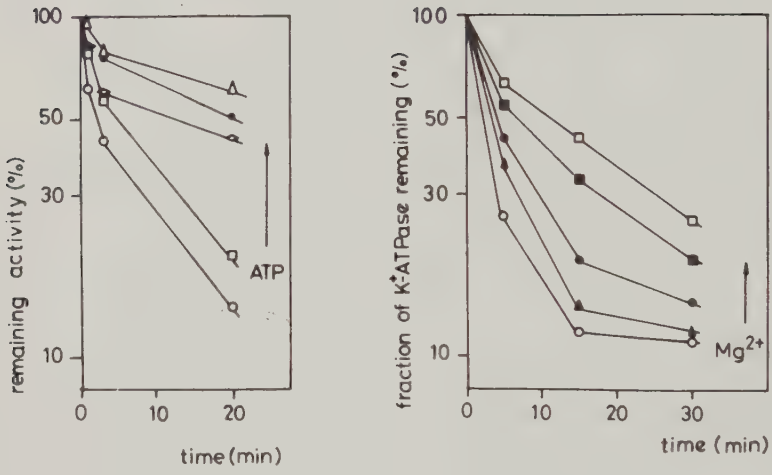

Figure 5. Effects of ATP (left figure) and magnesium (right figure) on the effect of tryptic inactivation of H,K-ATPase on the enzyme activity. Tryptic digestion was carried out with potassium alone (open circles) to which increasing concentrations of ATP or magnesium were added after which the activity was measured. From Helmich-de Jong et al, 1986 a.

Whether this site is additional to the high affinity binding site, or is a low-affinity form of the high affinity site only occurring under phosphorylation conditions has to be established. It is striking that also in Na,K-ATPase similar effects of ATP have been observed (Askari and Huang, 1982). In both enzymes the physiological role of this site is not clear.

Like in Na,K-ATPase various conformational states can be distinguished depending on the ligands present. The trypsin digestion studies of Helmich-de Jong et al (1987) indicate the presence of both a potassium- and an ATP-conformation. These conformational forms could be detected from both the products formed and the inactivation kinetics. With magnesium the trypsin digestion did not lead to the detection of a specific conformational form, since splitting products of both the potassium- and the ATP-form

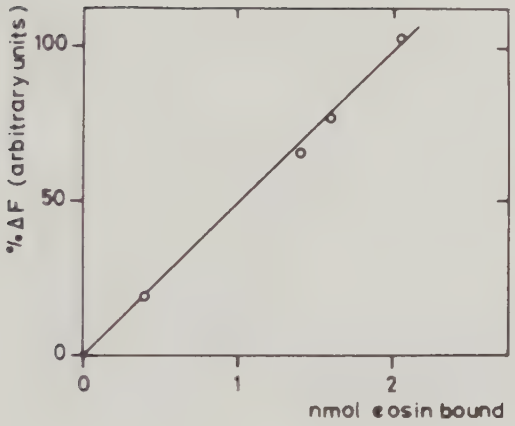

Figure 6. Relationship between fluorescence by eosin and binding to H,K-ATPase. The enzyme was incubated with 2 µM eosin at pH 7.5 with 0, 0.5, 1.0, and 2.0 mM magnesium Both fluorescence and binding increase with the magnesium concentration. From Helmich-de Jong et al, 1986 a.

could be detected in the presence of this cation. The effect of potassium present during trypsin digestion was counteracted by ATP as well as by magnesium (Fig 5).

With the fluorescent probe eosin, which binds to the ATP binding site various conformational states can also be distinguished (Helmich-de Jong et al, 1986 b). In the presence of magnesium more eosin is bound to H,K-ATPase leading to a higher fluorescence level (Fig 6). Both binding and fluorescence are counteracted by potassium, indicating that the magnesium and potassium conformation do differ. With this method an ATP-form of the enzyme cannot be distinguished.

Antagonistic effects between magnesium and potassium and between ATP and potassium have been observed before. The protecting effect of ATP against inactivation by butanedione is counteracted by potassium (Schrijen et al 1980). Magnesium lowers the stimulatory effect of potassium on the ADP/ATP exchange reaction and antagonizes the inhibitory effect of potassium on the steady-state phosphorylation level (Schrijen, 1981). Magnesium also antagonizes the lowering by potassium of the FITC fluorescence (Jackson et al 1983).

According to the nomenclature used for other transport ATPases it is logical to name the potassium-form E2. The above experiments indicate that both the magnesium-form and the ATP-form are of the E1 type, but are not identical.

In collaboration with E. Bamberg(Frankfurt a/M) membrane sheets of H,K-ATPase were attached to a black lipid membrane. Only in the absence of potassium but not in its presence an electrical signal could be elicited upon illumination of the compartment containing both caged ATP and H,K-ATPase. This signal could be blocked by vanadate and the H,K-ATPase inhibitor omeprazole indicating that it was specific. The fact that with H,K-ATPase in the presence of potassium no signal is found suggests that the reaction cycle of H,K-ATPase fits in general the Albers-Post scheme with the exception that the complete ATPase reaction is electroneutral. The positive signal in the absence of potassium suggests that the proton transport step is electrogenic and occurs in the E1P-E2P step (see also Bamberg et al, this meeting).

INHIBITORS

Specific inhibitors of H,K-ATPase are clinically very interesting substances. Overproduction of gastric acid can give rise to serious clinical problems. Most inhibitors of gastric acid secretion act as competitive agents on the histamine H2 binding site. However, since gastric acid secretion can also be stimulated by acetylcholine and gastrin and since the H,K-ATPase is the key enzyme in gastric acid secretion, use of a direct inhibitor of this enzyme would be preferable.

Several potent inhibitors of this enzyme have been reported (Table 1).

Table 1 Inhibitors of Gastric H,K-ATPase

name	reference
Picoprazole	Fellenius et al (1981)
Omeprazole	Larsson et al (1983)
Polyamines	Ray et al (1981)
Allylisothiocyanate	Takeguchi et al (1983)
Trifluoperazine	Im et al (1984)
Verapamil	ibid.
TMB-8	ibid.
SCH 32651	Scott et al (1985)
SCH 28080	ibid.
Ro 18-5364	Sigrist-Nelson et al (1986)

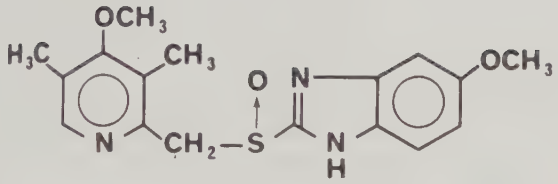

Figure 7. Structure of omeprazole.

Most of them, however, inhibit the enzyme only in the reagent tube or have to be studied in further detail. By far the best studied group of inhibitors is that of the substituted benzimidazoles, of which omeprazole is the mostly used one . Wallmark et al (1984) found in rat a good correlation between the inhibitory effects of omeprazole on the acid secretion rate, the H,K-ATPase activity and the phosphoenzyme level. Omeprazole has a number of properties which makes it a very suitable inhibitor.

Omeprazole is permeant and has a pKa value of 4, which causes its accumulation in an acid environment. Since acid spaces are present in the tubulovesicular system in the parietal cell and not in many other places in the body the drug will accumulate close to its site of action. In the acid environment omeprazole is thereafter converted via a spiro-intermediate into its active metabolite which is either a sulfenic acid or a sulfenamide (Lindberg et al, 1986). This active metabolite reacts with essential sulfhydryl groups present in H,K-ATPase leading to a inactivation of the enzyme. The activated form of omeprazole can also inhibit other enzymes like Na,K-ATPase. This mechanism of action explains why the effect of omeprazole can be antagonized by sulfhydryl group containing compounds, like glutathione (Wallmark et al 1984; Im et al 1985 b). The action of omeprazole in isolated gastric vesicles even requires proton transport (Lorentzon et al 1987). Only then a reliable value for the stochiometry of omeprazole binding (1.4-2 per mole phosphoenzyme) can be obtained. In vivo the effect of omeprazole appears to be irreversible (Im et al 1985 a), possibly since intracellularly the concentration of free sulfhydryl groups is not high enough. Omeprazole has been found to be a potent and long acting antisecretory drug in patients with the Zollinger-Ellison syndrome (Lamers et al 1984). In patients with duodenal ulcer, omeprazole accelerates healing and pain relief (Lauritsen et al 1985).

CONCLUDING REMARKS

During the last number of years it has become clear that H,K-ATPase and Na,K-ATPase are two enzymes which are not identical but closely related. Na,K-ATPase has been studied in more detail than H,K-ATPase, due to the fact that the former enzyme has a more general abundance and is known for a much longer time. H,K-ATPase can therefore be considered as Na,K-ATPase's stepsister. It is a relative but has not received yet so much dedication by the scientific community as her elder sister. Cinderella's prince has not arrived yet. Therefore investigators on H,K-ATPase have up till now learned much more from studies on Na,K-ATPase than oppositely. However, the comparison of the chemistry and the kinetics of the two enzymes will become profitable for a better understanding of Na,K-ATPase too. It has to be expected that further knowledge of the essential features of both enzymes and in particular of the protein chain of the catalytic subunit will lead to a molecular insight in the mechanisms of primary active transport and to the cause of diseases which are due to malfunctioning of these systems. Therefore comparison of H,K-ATPase and Na,K-ATPase will be important in near future too.

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OVERVIEW:

SUBUNIT INTERACTION AND CONFORMATIONAL STATES. Ca-ATPase and Na,K-ATPase compared

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INTRODUCTION

Understanding the molecular mechanisms of Na,K- and Ca-pumps requires elucidation of the role of subunit interaction. It is a classical idea that transient passages for ions can be formed at interfaces between peptide chains arranged in an oligomeric complex. Furthermore, interaction between subunits has been implicated in energy transduction by flip-flop mechanisms. On the other hand, the predicted tertiary structures of Na,K-ATPase and Ca-ATPase, comprising 3-5 hairpin loops through the membrane as well as possible additional intramembranal structures, show that a single peptide chain does provide enough membrane embedded domains to form a realistic alternative to oligomeric pump models.

In this review we will examine basic problems associated with definition of subunit structure of sarcoplasmic reticulum Ca-ATPase. It is not possible to determine the size of minimum structural or functional units in the membranous state, directly. After solubilization in detergent, molecular weight can be measured, but in this case the uncompartmentalized nature of soluble preparations precludes examination of vectorial transport. Valuable information as to the role of subunit interaction can, however, be obtained by characterization of enzymatic properties of soluble Ca-ATPase complexes. Based on current ideas about the elementary steps of Ca^{2+} transport (Fig. 1), particularly important questions are whether a single peptide chain in solution is able to occlude Ca^{2+} in E_1P, and whether it undergoes transition to the form of low Ca^{2+} affinity (E_2P), as required for energy transduction (de Meis, 1981). Furthermore, subunit interaction has been implicated in modulation of the reaction rate by ATP bound

with low affinity. The experimental foundation of this hypothesis will be examined in some detail. We will also be concerned with information defining the nature and extent of the conformational changes involved in Ca^{2+} transport. A fundamental idea underlying the E_1-E_2 notation used in Fig. 1 is that there are two major conformational pools characterized by opposite orientations of Ca^{2+} transport sites with respect to the bilayer. This may be contrasted with models in which a series of minor conformational changes lead to diffusion of ion through a pore-like structure. Experimental evidence suggesting that E_1-E_2 transition comprises movement of peptide mass relative to the bilayer will be discussed. This provides a mechanism by which transport can easily be understood in terms of transposition of ion binding sites, with a single peptide chain as the basic functional unit.

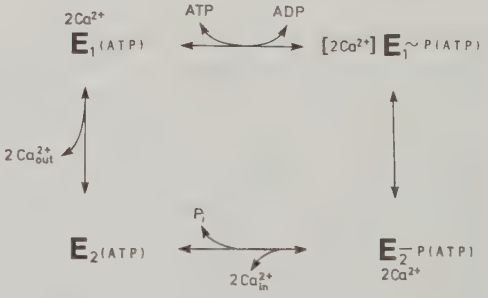

Figure 1. Minimum scheme for Ca-ATPase reaction cycle. E_1 states have outward (cytoplasmically) oriented Ca^{2+} sites with high affinity. E_2 states have inward (luminally) oriented Ca^{2+} sites of low affinity. Occluded Ca^{2+} is shown in brackets. E_1 accepts phosphate from ATP in a reversible reaction. E_2 reacts with P_i to form ADP insensitive phosphoenzyme. A "modulatory" ATP molecule is also shown.

SUBUNIT INTERACTION IN MEMBRANE BOUND Ca-ATPase

Existence of physical contacts between Ca-ATPase peptides in the sarcoplasmic reticulum membrane has been inferred from various kinds of measurement, including electron microscopy, fluorescence energy transfer, spin-labelling, covalent cross-linking and radiation inactivation, but the nature of these contacts is not well understood. The concentration of Ca-ATPase polypeptides in the membrane is quite high - about 30 000 peptides per μm^2, corresponding to approximately 5 mM. Therefore, a meaningful definition of subunit composition of the pump requires distinction between two major types of interaction:

1. Weak and non-specific associations between peptides, which occur merely as a result of the dense packing in the membrane. This type is not expected to play a central role in the mechanism of ion translocation, but it may well modify rate constants and be responsible for cooperative phenomena observed in kinetic and equilibrium binding studies.

2. Specific associations leading to formation of a well defined minimum structural and functional unit of size higher than monomer.

One illustrative result of the first type of interaction is the formation of bidimensional crystalline arrays after conformational stabilization with vanadate or lanthanides (Dux et al., 1985). This requires a peptide density of more than 1000-2000 per μm^2 (Dux, 1985). Smaller peptide clusters of varying size (Franzini-Armstrong et al., 1985) as detected by electron microscopy of non-crystalline membranes may also result from interaction of the first type. Existence of the second type of subunit interaction is much more open to question.

Analysis of Bidimensional Crystals

Two types of crystals have been characterized (Dux et al., 1985). These appear to be associated with E_1 and E_2 conformations, respectively. The enzyme in either one of the crystal lattices is unable to hydrolyze ATP, but reversible interconversion between E_1 and E_2 crystal forms can be demonstrated.

In vanadate induced crystals (E_2 conformation), the crystallographic unit cell contains two peptide chains. The hydrophilic protrusions of individual peptides are in close contact, two by two forming bridge-like structures on the cytoplasmic surface. However, the hydrophobic domains of the peptide pairs spread apart as they span the bilayer, and interact with the intramembranal domains of neighbouring pairs (Ho et al., 1986). Thus, it is not possible to define a dimeric structural unit in an unambiguous way. It is not straight forward to decide which type of interaction (between hydrophobic or hydrophilic domains) that might be implicated in formation of dimers in non-crystalline functional membranes.

In lanthanide induced crystals (E_1 conformation) the minimum asymmetric unit is a single peptide. This is an important

information which appears to eliminate the existence of a well defined oligomeric unit, in this condition. However, the close relationship between crystal structure and conformational state of Ca-ATPase (E_1: monomer, E_2: dimer) raises the question if alteration between monomer and dimer could be involved in the transport reaction. Note, on the other hand, that in contrast to Ca-ATPase crystals, there is no simple relationship between unit cell structure and conformation in Na,K-ATPase crystals. Both monomeric and dimeric crystal types can be obtained from E_2 states (Jørgensen et al., 1982).

Reconstituted Vesicles

The idea that Ca^{2+} transport depends on transient formation of dimers in the membrane can be tested: if it were true, a significant reduction of peptide density should lead to a diminished transport competence. By reconstitution of soluble Ca-ATPase with excess phospholipid and subsequent sucrose density centrifugation, it has been possible to prepare vesicles containing as little as 2-3 peptides (i.e. with a peptide density close to 100 per μm^2) (Andersen et al., 1983). These vesicles were shown to transport Ca^{2+} with a similar specific activity as that of native sarcoplasmic reticulum vesicles. This suggests that the transport mechanism does not involve a shift between different peptide selfassociation states.

Examination of the reconstituted vesicles by freeze-fracture electron microscopy has shown that the dispersed pump units give rise to distinct intramembranous particles of a similar size as that of most intramembranous particles observed in native sarcoplasmic reticulum (diameter 60-90 Å, depending on the technique of metal shadowing). It is not possible to assign this size to a certain subunit structure, due to uncertainty with respect to the exact mass of the intramembranous part of Ca-ATPase and with respect to the relative contributions of protein, lipid annulus, and metal shadowing layer to the observed particle structure.

Radiation Inactivation

In principle studies of target size, using radiation inactivation, should permit definition of the minimum functional unit of Na,K- and Ca-pumps in the membranous state. In practice, results obtained by this method are often hard to interpret (Jensen and Ottolenghi, 1985). In one study of frozen samples of Ca-

ATPase (Hymel et al., 1984), the decay constant describing disappearance of ATPase activity as a function of radiation dose was found to correspond to that of a dimer, but a similar target size was observed for fragmentation of the single peptide chain as seen on SDS gels. This suggests that either radiation energy is dissipated between two polypeptides in a dimeric complex, despite the absence of covalent bonds (which is somewhat unexpected, cf. Kempner and Miller, 1983) or, alternatively, that the target is a monomer, and for some as yet unidentified reason, the decay constant overestimates the peptide size. An important problem is that the contribution of the lipid annulus to target size is unknown. Furthermore, the role of secondary attack by products of radiation damage remains to be elucidated. The use of watersoluble enzymes as standards for membrane proteins has not yet been validated. A comparison of Ca-ATPase with Na,K-ATPase shows that the two cation pumps have similar target sizes (with possible allowance for a minor difference corresponding to the β subunit). This may be suggestive that they contain the same number of protomers, but even this conclusion should be regarded with suspicion as long as the ambiguities mentioned above exist.

DETERGENT SOLUBILIZED Ca-ATPase

The problems involved in delineating subunit structure of the Ca-pump **in situ** has focused attention on characterization of detergent solubilized enzyme. Ca-ATPase can be solubilized with retention of activity in a variety of detergents, ionic as well as non-ionic, e.g. deoxycholate, Triton X-100, $C_{12}E_8$ (octaethyleneglycol monododecyl ether) and lysophosphatidyl choline. This permits molecular weight determination by rigorous physical methods, such as analytical ultracentrifugation (see Møller et al., 1986 for an exhaustive review of methods). An important complication is that soluble enzyme forms tend to be unstable and undergo denaturation and irreversible aggregation - especially after removal of phospholipid. However, denaturation can be delayed by addition of polyhydroxy componds such as sucrose or glycerol, as well as appropriate ligands that bind tightly to the enzyme (Ca^{2+}, nucleotides, vanadate). Several years ago, work initiated in Dr. Tanfords laboratory (le Maire et al., 1976; Dean and Tanford, 1978; Jørgensen et al., 1978) established that the minimum unit required for full ATP hydrolysis activity is a monomer. Later, a detailed characterization of enzymatic and structural properties of monomeric Ca-ATPase was undertaken (Møller et al., 1982; Andersen et al., 1982, 1985 a,b, 1986b; Vilsen and Andersen, 1986, 1987).

Reversible Monomer-oligomer Equilibrium

Solubilization of Ca-ATPase in predominantly monomeric form in $C_{12}E_8$ requires that the detergent to protein concentration ratio is well above 1:1 (w/w). In order to study the reversible monomer-oligomer equilibria that exist at lower ratios, experiments must be carried out before any irreversible changes in selfassociation state take place as a result of denaturation. The advent of HPLC columns for rapid high resolution size exclusion chromatography has recently made this possible. After the relation between column elution positions and molecular weight has been established by analytical ultracentrifugation, the most convenient way of determining equilibrium constant for peptide selfassociation is by large zone HPLC (Andersen et al., 1986b). In this technique, the sample size is large relative to the volume of the column, so that the protein does not become diluted during passage. Thereby, it is possible to measure the averaged partition coefficient in the column as a function of protein concentration (Fig. 2). This provides a K_d value for dimerization ranging from 10^{-3} to 10^{-2} mM, depending on detergent and phospholipid concentrations. The tendency for selfassociation increases in presence of phospholipid, and at low detergent concentration. The conclusion that can be derived by extrapolation of results such as those shown in Fig. 2 to the lipid and protein concentrations of sarcoplasmic reticulum (if possible) is consistent with observation of frequent interpeptide associations in situ. Studies of oligomers in detergent do however not provide a more unequivocal definition of subunit structure, than do the previously described studies of membrane bound enzyme. It has not been possible to isolate a well defined homogenous oligomer, except after denaturation or covalent cross-linking. Active dimers of Ca-ATPase solubilized in $C_{12}E_8$ have always been found to be in rapid equilibrium with monomers or higher oligomers. This is perhaps a major distinction from soluble Na,K-ATPase, which has been isolated in active form as pure dimer (Esmann et al., 1980).

The existence of reversible equilibria between monomers and oligomers in the soluble state raises the question if the apparently monomeric Ca-ATPase preparations obtained at high detergent to protein concentration ratios, actually remain unassociated during substrate utilization. Compelling evidence that they **do** has been obtained by "active enzyme centrifugation" (Martin, 1983) and by HPLC during enzymatic turn over (Andersen and Vilsen, 1985). This is illustrated by the data shown in Fig. 2B. At low protein concentration where the enzyme is monomeric, the elution position is unaffected by addition of ATP. At high protein concentration

where reversible selfassociation occurs in absence of nucleotide, addition of ATP to initiate the catalytic cycle results in shift of the equilibrium towards monomer, rather than towards dimer. A similar effect as with ATP was observed with vanadate which stabilizes the E_2 conformation (Andersen and Vilsen, 1985). Thus, there is no reason for expecting that E_2 forms have a higher tendency for selfassociation in detergent than E_1 forms.

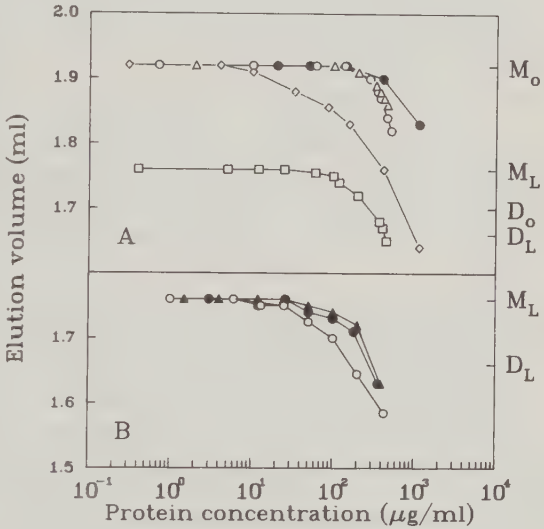

Figure 2. Monomer-dimer equilibrium of $C_{12}E_8$ solubilized Ca-ATPase studied by large zone HPLC. Soluble enzyme was injected into a TSK GSWP HPLC column, and the elution volumes corresponding to leading boundary centroid positions were determined (for methods see Andersen et al., 1986b). (A) Experiments performed at 20°C. (●), delipidated Ca-ATPase at 2.0 mg $C_{12}E_8$/ml; (○), Ca-ATPase with endogenous lipid at 2.0 mg $C_{12}E_8$/ml; (△), 30% glycerol present; (◇), delipidated Ca-ATPase at 0.1 mg $C_{12}E_8$/ml; (□), Ca-ATPase in presence of 1.0 mg dioleoyl lecithin/ml and 2.0 mg $C_{12}E_8$/ml. (B) Experiments performed at 2°C. Same medium as in A (□) except that ATP (25 μM and 1.0 mM) was added in (●) and (▲), respectively. "M_L" and "M_o" indicate elution positions of monomeric Ca-ATPase in presence and absence of 1 mg phospholipid/ml, respectively. "D_L" and "D_o" indicate elution positions of covalently cross-linked dimeric Ca-ATPase in presence and absence of 1 mg phospholipid/ml, respectively.

Site Stoichiometry

A classical question for Na,K- and Ca-pumps is why we measure only half of the ligand binding capacity, expected from determination of protein concentration. One possibility is that subunit interaction in a dimer hinders simultaneous binding of ligand to both chains. Two results with soluble Ca-ATPase speak against such "half-of-the-sites reactivity":

1. When Ca-ATPase is solubilized in monomeric form under conditions where denaturation is too slow to be important, and

insoluble residue is not removed, the capacity for ligand binding (Ca^{2+}, phosphate, vanadate) is found to be the same (within ±10%) as that of the parent membraneous preparation (Andersen et al., 1982, 1985b, 1986b).

2. Purification of sites to 6-7 nmol EP/mg and 12-15 nmol bound Ca^{2+}/mg can be achieved after detergent solubilization, by salt fractionation or HPLC (MacLennan et al., 1971; Barabin et al., 1984; Andersen et al., 1986b). Most of the inactive protein removed consists of denatured Ca-ATPase which is aggregated, even in SDS (Barabin et al., 1984).

The only logical conclusion is that each non-denatured peptide possesses one available phosphate site and two Ca^{2+} sites. The reason for the presence of denatured protein is not known. An effect of high pressure, as developed during preparative centrifugation, has been proposed (Varga et al., 1986).

Ca^{2+} Occlusion And Energy Interconversion in Monomeric Ca-ATPase

Soluble monomeric Ca-ATPase is able to undergo the transition between ADP-sensitive and -insensitive phosphoenzymes and synthesizes ATP from added ADP and P_i by reversal of the cycle in Fig. 1 (Andersen et al., 1985a,b; Martin et al., 1984). An important problem is whether events in the catalytic site of soluble monomers are appropriately coupled with structural changes in Ca^{2+} sites.

In the sarcoplasmic reticulum membrane, formation of ADP sensitive phosphoenzyme, E_1P, results in "occlusion" of bound Ca^{2+}. This is analogous to occlusion of Na^+ in the phosphorylated form of Na,K-ATPase. The occluded state is likely to play a key role as a transport intermediate in the pathway of Ca^{2+} from one side of the membrane to the other. As seen in Fig. 3, $C_{12}E_8$ solubilized monomeric Ca-ATPase phosphorylated by use of the β,γ-bidentate Cr(III) complex of ATP (CrATP) retains bound Ca^{2+} during a small zone HPLC experiment (Vilsen and Andersen, 1986). The half-life for Ca^{2+} exchange was found to be of the order of hours. With CrATP as substrate the phosphoenzyme isomerization to E_2P is inhibited (Vilsen and Andersen, 1987), and this is probably the reason why deocclusion is slow enough to permit the measurement in Fig. 3. This data indicates that neither subunit interaction nor the presence of a lipid bilayer are required for occlusion of Ca^{2+}.

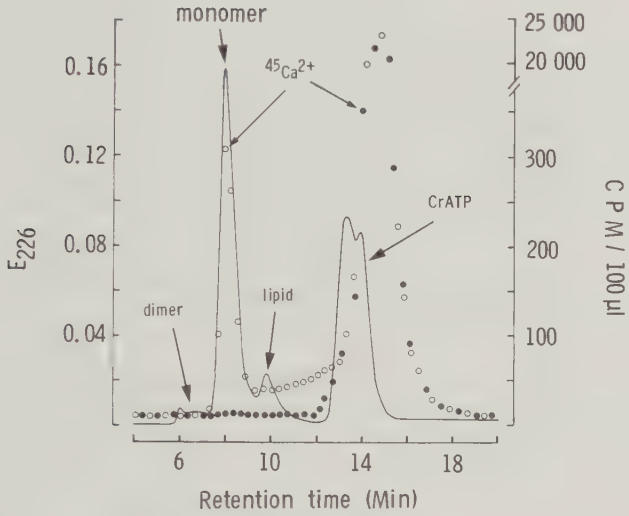

Figure 3. CrATP induced Ca^{2+} occlusion in soluble monomeric Ca-ATPase. (○), soluble monomer was reacted with CrATP in presence of $^{45}Ca^{2+}$ and then subjected to size exclusion HPLC in presence of a large excess of $^{40}Ca^{2+}$ to permit isotope exchange in non-occluded sites. (●), control experiment with no CrATP added.

Evidence that the CrATP induced Ca^{2+} occlusion occurs at specific high affinity Ca^{2+} transport sites is the following (Vilsen and Andersen, 1987): (a) Activation of Ca^{2+} occlusion takes place in the same Ca^{2+} concentration range (10^{-8}-10^{-6} M) as activation of ATP hydrolysis and Ca^{2+} transport, and is influenced by H^+ and Mg^{2+} in the same way as equilibrium Ca^{2+} binding to high-affinity transport sites. (b) The stoichiometry is 2 mol Ca^{2+} occluded per mol Ca^{2+} dependent phosphoenzyme formed - in keeping with the established transport stoichiometry. (c) At saturating Ca^{2+}-concentrations, binding of Mg^{2+} increases the rate of Ca^{2+} occlusion. This shows that Ca^{2+} occlusion takes place at sites distinct from the unspecific divalent cation sites normally occupied by Mg^{2+}.

In the E_1-E_2 reaction cycle (Fig. 1) deocclusion and release of Ca^{2+} on the luminal side of the membrane is coupled with transition of E_1P to E_2P. An essential aspect of energy transduction is that this step leads to more than 1000-fold reduction of Ca^{2+} affinity relative to the Ca^{2+} affinity of E_1. As seen in Fig. 4, addition of ATP to soluble monomeric Ca-ATPase

previously stabilized as E_1Ca_2 at micromolar free Ca^{2+}, leads to a temporary net release of bound Ca^{2+}. This is closely matched by change of ADP sensitivity of phosphoenzyme and rise of fluorescence from TNP-ADP (Andersen et al., 1985a). The latter compound probably reports on change in hydrophobicity of the catalytic site associated with E_1P-E_2P transition (de Meis, 1981; Davidson and Berman, 1987). By titration of the extent of net release of Ca^{2+} at various Ca^{2+} concentrations, it has been possible to show that the increase of chemical potential of bound Ca^{2+} caused by E_1P-E_2P transition in monomer is of the same magnitude as that observed with membrane bound enzyme preparations (Andersen et al., 1985b).

The combined evidence from the experiments shown in Fig. 3 and Fig. 4 indicates that the machinery required for coupling of catalytic events with structural changes in Ca^{2+} transport sites is present within one peptide.

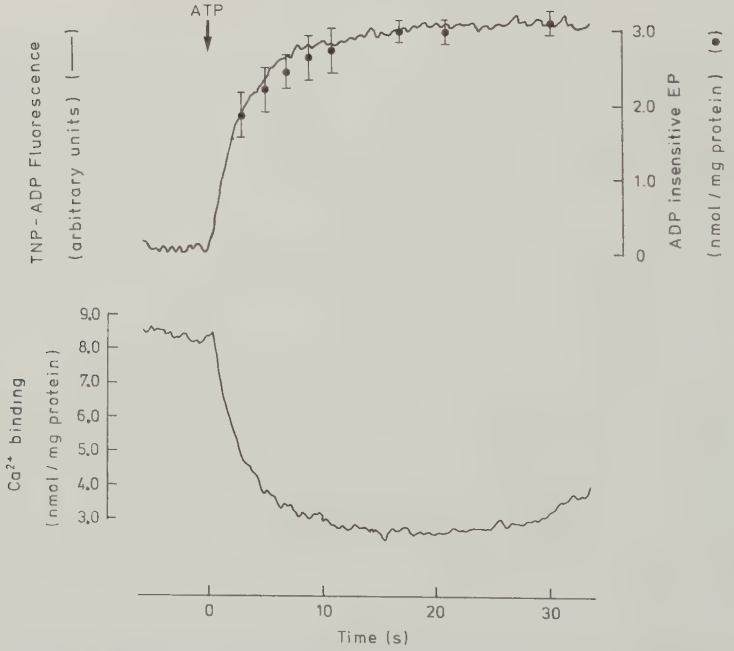

Figure 4. Energy transduction in soluble monomeric Ca-ATPase. The monomeric enzyme was stabilized in the E_1Ca_2 state, and ATP was then added as indicated to initiate phosphorylation and subsequent transition to E_2P. Upper trace: appearance of E_2P. Lower trace: dissociation of Ca^{2+}.

ATP MODULATION OF REACTION RATES

Binding of ATP to the catalytic site of the E_1Ca_2 form of Ca-ATPase occurs with one apparent K_d of 3-20 μM as measured by ATP concentration dependence of initial phosphorylation rates. However, the P_i liberation rate as a function of ATP concentration has a biphasic course with a secondary rise in the millimolar ATP concentration range. Several explanations may be advanced to account for this complex behaviour:

(A) Interaction between catalytic sites on subunits in an oligomer (Froehlich and Taylor, 1976; Plesner, 1987).

(B) Existence in the membrane of distinct non-interconvertible peptide populations with different V_{max} and K_m.

(C) Acceleration of a rate limiting step in the reaction cycle induced by binding of ATP to a low affinity regulatory site (Dupont, 1977; Carvalho-Alves et al., 1985).

(D) The affinity of the catalytic site for ATP or MgATP alternates between high and low during the catalytic cycle (Moczydlowski and Fortes, 1981). As a consequence of the "principle of linked functions", this will imply that the rate constant for transition between forms of different ATP affinity depends on occupancy of the site in its low affinity form (Reynolds et al., 1985).

At present there is no unequivocal evidence defining one of the above mentioned possibilities as **the** mechanism. In fact it is likely that more than one mechanism is operating (Champeil et al., 1986). In the following we will list some points which should be taken into considerance in evaluation of the relative importance of each of the proposed possibilities:

1. A combination of micromolar ATP with millimolar non- or slowly hydrolyzable ATP analogue (AMPCPP, AMPPCP) reproduces the turn over activation observed with millimolar ATP (cf. Dupont, 1977; Cable et al., 1985). This suggests that activator ATP may be different from the ATP molecule being hydrolyzed, in contradiction with mechanism (B).

2. In contradiction with mechanism (A), labelling of a single lysine residue in the catalytic site with FITC (fluorescein isothiocyanate) results in an inhibition of ATP binding and ATPase activity, which is linearly dependent on the amount of label incorporated. Modification of a fraction of the peptides in a membrane preparation with FITC leaves substrate dependence of the ATP hydrolysis catalyzed by the remaining half of the peptides undisturbed (Andersen et al., 1982; Cable et al., 1985).

3. The ATP induced dissociation of oligomers observed in large zone HPLC studies of soluble Ca-ATPase is saturated at micromolar ATP concentration (Fig. 2B). If destabilization of dimers with dual occupation of catalytic sites had been involved in the biphasic activation pattern (mechanism (A)), a significant further dissociation of the soluble peptides would have been expected to occur in the millimolar ATP concentration range.

4. It has not been possible to prove existence of a low affinity regulatory ATP site convincingly (mechanism (C)). Some workers observe binding of nucleotide in excess of one mol per mol catalytic site (Dupont, 1977; Carvalho-Alves et al., 1985), whereas others do not (Champeil and Guillain, 1986). In the former case the specificity of the extra ATP binding is questionable and the number of low affinity sites is ill-defined.

5. ATP induced enhancement of the E_2-E_1 transition rate (Mechanism (D)) has been demonstrated in kinetic experiments (cf. Scofano et al., 1979; Guillain et al., 1981). However, the observed $K_{0.5}$ for this effect is too low to account for modulation of the overall reaction rate in the millimolar ATP concentration range .

6. Equilibrium binding data suggest that in the E_2 form of Ca-ATPase, the catalytic site has a similarly high affinity as in the E_1Ca_2 form (cf. Andersen and Møller, 1985). This contrasts with studies of Na,K-ATPase. The measured ATP affinity of the E_2K form of this enzyme is low, in agreement with the requirement defined by kinetic simulations according to model (D).

7. In experimental conditions where the biphasic activation of ATP hydrolysis is most pronounced (e.g. neutral pH, presence of 0.1 M KCl), the E_1P-E_2P transition is the slowest of the proposed steps in the reaction cycle (Champeil et al., 1986). This step is therefore a likely candidate as target for modulation of the overall reaction rate.

8. As directly demonstrated in Fig. 5, millimolar ATP enhances the E_1P-E_2P transition rate. The magnitude of the relative increase of the rate and the $K_{0.5}$ for this effect (approximately 1 mM) are in accordance with the kinetic parameters for the low affinity activation phase of overall ATP hydrolysis. The effect of millimolar ATP is observed also in soluble monomeric preparations (Fig. 5, lower panel). The activating ATP molecule therefore binds to the same peptide as the one bearing the covalently attached phosphate. There is indirect evidence that ATP can bind to the catalytic site of phosphoenzyme after departure of ADP (Andersen and Møller, 1985; Champeil and Guillain, 1986), but further experimentation is required to decide if the affinities of catalytic sites of E_1P and E_2P differ (cf. mechanism (D)).

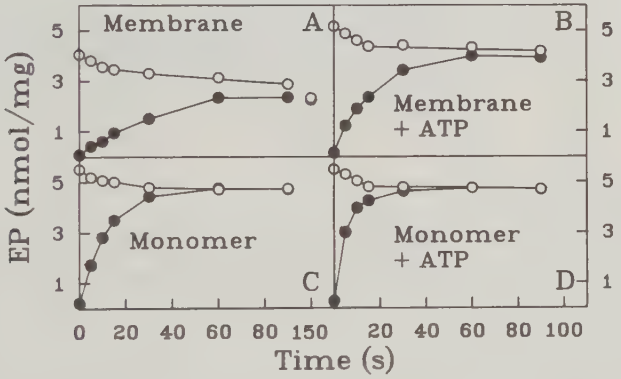

Figure 5. Enhancement of E_1P-E_2P transition rate by millimolar ATP. Membrane bound (A, B) or soluble monomeric Ca-ATPase (C, D) was phosphorylated with 25 μM $AT^{32}P$. At the time indicated as zero, EGTA was added to stop phosphorylation and observe transition to ADP insensitive phosphoenzyme. In B and D, 1 mM non-radioactive ATP was added with EGTA. (○), total EP; (●), ADP insensitive EP.

An attractive hypothesis is that E_1P is destabilized by unfavourable interaction between the aspartyl phosphate and ATP bound in the vicinity. It is also possible that the simultaneous presence of two Mg^{2+} ions (one tightly bound with the phosphate and one interacting with ATP) plays a role (cf. Andersen and Møller, 1985). Movement of peptide segments during the E_1P-E_2P conformational change (see below) may involve separation of aspartyl phosphate from the nucleotide site, leading to abolition of the destabilizing effect. Such a separation has previously been proposed for Na,K-ATPase (Jørgensen et al., 1982).

STRUCTURAL CHARACTERIZATION OF CONFORMATIONAL CHANGES

Movement of Ca^{2+} Sites

Hasselbach et al. (1983) have obtained evidence that stabilization of E_2 with vanadate induces appearance of new Ca^{2+} binding sites on the luminal surface of the membrane concomitantly with disappearance of cytoplasmic sites. The luminal sites created have much lower affinity but a higher capacity than their cytoplasmic counterpart. There is considerable experimental uncertainty involved in measuring low affinity binding, but if this result can be confirmed, it is tempting to speculate that the same coordinating groups contribute to cytoplasmic high affinity Ca^{2+} sites in E_1 and luminal low affinity sites in E_2. The lower affinity and higher number of sites in E_2 may be accounted for by an increased distance between some of the coordinating groups after reorientation.

Proteolytic Cleavage

Proteolytic cleavage patterns of Ca-ATPase provide direct evidence for a conformational change (Andersen and Jørgensen, 1985; Andersen et al., 1986a). It can be seen in Fig. 6 that the cleavage site giving rise to A_1 and A_2 tryptic fragments ("T_2 site" at Arg 198) is more exposed to trypsin in E_1 and E_1P states than in E_2 and E_2P. This provides a structural distinction between two major conformational pools. The conformation sensitive split is located in the second cytoplasmic domain ("transduction domain", cf. Brandl et al., 1986) between the second and third transmembrane segments identified in hydroplots (Fig. 7). This is the same domain where Na^+ induced tryptic and chymotryptic splits are found in Na,K-ATPase (C_3 and T_3). Thus, it seems to be a common feature of the two cation pumps that this part of the chain is exposed to cleavage in E_1 forms and protected in E_2.

It is of importance that although the conformation sensitive split is located rather close to putative Ca^{2+} binding sites on amphipatic helixes (cf. Fig. 7), phosphorylation with concomitant Ca^{2+} occlusion (i.e. formation of E_1P from E_1) has no influence on the cleavage rate. This suggests that in contrast to E_1-E_2 and E_1P-E_2P transitions, occlusion may be a very local event, possibly involving removal of the last few water molecules in the first coordination sphere of bound Ca^{2+}. Alternatively, the speculation about position of Ca^{2+} binding sites is wrong.

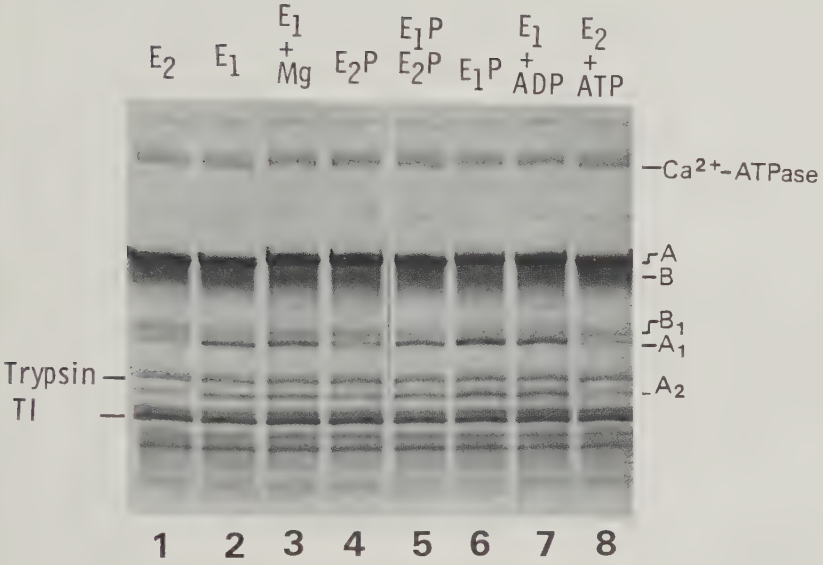

Figure 6. SDS gelelectrophoresis showing tryptic cleavage patterns of Ca-ATPase in various functional states. The predominant enzyme forms present during proteolysis are indicated above the lanes. Titration of the steady state distribution of E_1P and E_2P was performed by varying the Ca^{2+}/Mg^{2+} concentration ratio present with ATP (cf. Andersen et al., 1985a,b).

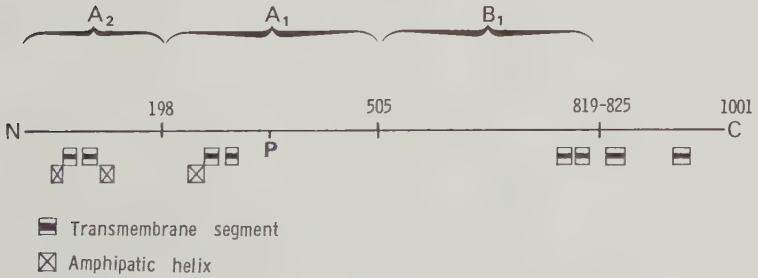

Figure 7. Linear map of Ca-ATPase peptide showing the relation of major tryptic cleavage sites to the position of transmembrane segments identified in hydroplots. Also shown, are the proposed amphipatic helices, which carry charged residues thought to be involved in formation of Ca^{2+} sites (Brandl et al., 1986).

Fig. 6 also shows that addition of ATP to enzyme stabilized in E_2 by absence of Ca^{2+} has little effect on the appearance of A_1 and A_2 fragments on the gel (compare lanes 1 and 8). This constitutes an important difference from the well known nucleotide effect on E_1-E_2 equilibrium of Na,K-ATPase, but is in accordance with binding measurements indicating that E_1 and E_2 forms of Ca-ATPase differ much less, if at all, with respect to nucleotide affinity than do their Na,K-ATPase counterparts.

Protection of the tryptic cleavage site at Arg 198 in E_2 forms also takes place in $C_{12}E_8$ solubilized monomeric Ca-ATPase (Andersen et al., 1985a). This shows that prevention of trypsin access to the bond is caused by a change in peptide structure **per se** rather than by a change of subunit interaction or a change of lipid-protein interaction.

Transposition of Peptide Mass

Comparison of ultrastructures of vanadate incubated membrane and membrane in which Ca-ATPase is in E_1 form suggests that E_1-E_2 transition may involve redistribution of peptide mass from the cytoplasmic surface to the inner half of the bilayer (Hasselbach et al., 1983; Coan et al., 1986). This kind of movement has also been inferred from time-resolved X-ray studies (Blasie et al., 1985). We have examined the reactivity of the hydrophobic photoactivable label TID (trifluoromethyl [^{125}I]iodophenyldiazirine) in four different functional states: E_1, E_2, E_1P, and E_2V (Andersen et al., 1986a). The relative amount of label incorporated in Ca-ATPase protein after illumination of sarcoplasmic reticulum vesicles is 10-20% higher in E_2 forms than in E_1. The largest difference was observed between E_1 stabilized in the phosphorylated form with Ca^{2+} + CrATP and E_2 stabilized with vanadate. After separation of tryptic fragments by HPLC in SDS, the major part of the preferential hydrophobic labelling of E_2 was found to be located on the A_1 fragment (residues 199-505, cf. Fig. 7). This fragment includes the third and the fourth transmembrane segments as well as the phosphorylation site (Asp 351). Assuming that the label distributes evenly on peptide segments in contact with lipid, and that at least 200 residues are buried in the membrane phase in E_1, it can be calculated that the difference between hydrophobic labelling of E_1 and E_2 forms corresponds to movement of 38 residues into the bilayer. A model for such a movement involving initial rotation of amphipatic helices to expose their hydrophobic surfaces to water has been proposed (Brandl et al., 1986).

CONCLUSIONS

1. There is no unequivocal evidence for existence of a minimum structural or functional unit of size higher than monomer in the native membrane.

2. Monomers in solution possess Ca-ATPase activity and are able to occlude Ca^{2+} in E_1P and couple E_1P-E_2P transition with an increase of chemical potential of bound Ca^{2+}.

3. Modulation of the reaction rate by millimolar ATP does not depend on subunit interaction.

4. The structural information suggests that transition between E_1 and E_2 states involves a significant change of tertiary structure, resulting in a deeper immersion of the peptide in the bilayer. This is in accordance with (but does not prove) a model in which Ca^{2+} is carried across the membrane by reorientation of binding sites within a single peptide chain.

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OVERVIEW: ENERGY TRANSDUCTION IN BIOLOGICAL MEMBRANES. ROLE OF SOLVATION ENERGY.

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In water the hydrolysis of an acyl phosphate residue, ATP or inorganic pyrophosphate is accompanied by a large change in free energy (George et al. 1970; Hayes et al., 1978). During the catalytic cycle of enzymes involved in energy transduction there are steps in which the hydrolysis of these compounds is accompanied by only a small energy change. The change in the energy of hydrolysis of the phosphate compounds that occurs on the enzyme surface appears to be crucial to the mechanism of energy transduction in the living cell. Some of the enzymes in which this phenomenon is observed are the Ca^{2+}-ATPase of sarcoplasmic reticulum (Masuda and de Meis, 1973; de Meis and Vianna, 1979; de Meis, 1981; de Meis et al. 1982; Tanford, 1984), the (Na+K)-ATPase of plasma membrane (Post et al., 1975; Taniguchi and Post, 1975), F_1 ATPase of mitochondria and chloroplasts (Boyer et al., 1973, 1977; Rosen et al. 1979; Grubmeyer et al. 1982; Tanford, 1983), myosin (Eisenberg and Hill, 1985), inorganic pyrophosphatase (Janson et al., 1979; Springs et al. 1981) and the H^+-ATPase of yeast plasma membrane (Goffeau and Slayman, 1981; Amory et al., 1982). A commo feature of these enzymes is that they undergo a conformational change during the catalytic cycle. Recently it has been proposed

that the conformational change leads to a small decrease in the water activity within the catalytic site and that this hydrophilic-hydrophobic transition would promote a decrease in the energy of hydrolysis of the phosphate compound (de Meis, 1981, 1984, 1986; de Meis et al. 1980, 1982; de Meis and Inesi, 1982; Behrens and de Meis, 1985;). The phosphate compound would bind to the enzyme when the catalytic site is hydrophilic and after binding it would remain a high energy molecule but would not be hydrolyzed. Following the conformational change, the catalytic site would become hydrophobic, there would be a large decrease in the Keq for hydrolysis and the phosphate compound would be cleaved. In this formulation the energy of hydrolysis of the phosphate compound would vary depending on the relative solvation energies of reactant and product (George et al., 1970; Haynes et al., 1978; de Meis, 1981).

This proposal is based on the following data.

(1) Inorganic phosphate, Pi, is highly soluble in water and poorly soluble in organic solvents. If the catalytic site of the enzyme is hydrophobic, then the partition of Pi from the assay medium into the catalytic site should be facilitated when the difference in hydrophobicity between these two compartments is decreased. Accordingly, it was found that the addition of organic solvents to the assay medium caused a large decrease of the Pi concentration required for half-maximal synthesis of an acyl phosphate residue at the catalytic site of the Ca^{2+}-ATPase (de Meis, et al., 1980, 1982; de Meis, 1981, 1986; Champeil et al., 1985), of tightly bound ATP by the F_1 ATPase, (Sakamoto, 1984; de Meis, 1986; Gomez-Puyou et al., 1986; Kandpal et al., 1987), for half maximal rate of Pi : PPi exchange catalyzed by yeast inorganic pyrophosphatase (de Meis, et al. 1986) and half maximal rate of Pi : ATP exchange catalyzed by the H^+-ATPase of yeast plasma membrane (de Meis, et al., 1987).

(2) Experiments performed with fluorescent probes indicate that there is a change of solvent structure at the catalytic site of the Ca^{2+}-ATPase during the catalytic cycle (Watanabe and Inesi, 1982; Kurtenbach and Verjovski-Almeida, 1985; Highsmith, 1986).

(3) Andersen et al. (1986) observed that there was an increase of the hydrophobic labeling of the sarcoplasmic reticulum ATPase with the photoactivable reagent trifluoromethyl (^{125}I) iodophenyl-diazarine when the enzyme was stabilized in the form that binds Pi as compared with the form which binds ATP. The preferential hydrophobic labeling was found to be located in the same tryptic fragment where the catalytic site of the enzyme is located.

(4) In mixtures of organic solvents with water, the observed energy of hydrolysis of pyrophosphate is up to 5 kcal/mol smaller than that measured in totally aqueous medium (de Meis, 1984; de Meis, et al., 1985; Wolfenden and Williams, 1985).

(5) Chromatophores of Rhodospirillum rubrum contain a membrane-bound pyrophosphatase that is able to catalyze the synthesis of pyrophosphate in the light. Synthesis is driven by the energy derived from the electrochemical H^+ gradient generated across the membrane of the chromatophores during illumination. The mechanism by which the energy of the electrochemical H^+ gradient is used for the synthesis of pyrophosphate is still unknown. In a recent report (Behrens and de Meis, 1985) the synthesis of pyrophosphate by chromatophores in the light was compared with that catalysed by soluble yeast inorganic pyrophosphatase in mixtures of organic solvents and water. The aim was to explore the possibility that a decrease of water activity may play a role in the mechanism by which the energy derived from the electrochemical H^+ gradient is used for the synthesis of pyrophosphate in chromatophores of R. rubrum. It was observed that in the absence of a H^+

gradient, the soluble yeast pyrophosphatase catalyzes the synthesis of pyrophosphate in an amount similar to that obtained with the use of chromatophores (H^+ gradient) under illumination when the water activity of the medium was decreased by the addition of organic solvent. For a given Pi concentration the maximal amount of pyrophosphate synthesized by chromatophores varies, depending on the pH and $MgCl_2$ concentration in the medium. A similar pH and $MgCl_2$ dependence was observed for the synthesis of pyrophosphate catalysed by soluble pyrophosphatase in the presence of organic solvent.

(6) Inhibition by hydrophobic molecules. According to the hypothesis proposed , the catalytic site of enzymes involved in energy transduction have a hydrophobic character when they are in the conformation in which the enzyme binds Pi. In this case it should be expected that hydrophobic molecules would compete with Pi and inhibit the enzyme activity because they readily partition from the assay medium (hydrophilic) into the hydrophobic environment of the catalytic site. This inhibition should be antagonized by organic solvents. The solvent would decrease the difference in hydrophobicity between the active site and the medium and thus decrease the force that drives a hydrophobic molecule out of an hydrophilic medium into an hydrophobic environment. The ATPase activity of soluble F1 ATPase was found to be inhibited by the Fe^{2+} chelate of batophenantroline and by anticalmodulin substances such as trifluoperazine and calmidazolium (de Meis et al., 1987). These molecules are poorly soluble in water and freely soluble in organic solvents. The inhibition promoted by these substances is antagonized both by Pi and organic solvents. Essentialy the same results were obtained with the Ca^{2+} ATPase (de Meis et al. 1987).

(7) Activation energy. An apparent paradox to this proposal is that the phosphate compound is cleaved when the catalytic site becomes hydrophobic. Because water is one of

the reactants for the hydrolytic reaction, intuitively one would expect that a decrease in water activity should impair the cleavage of the phosphate compound and not facilitate it. We have measured the spontaneous hydrolysis of acetyl phosphate and ATP in alkaline and acid solutions with different water activities (de Meis, 1982). The aim was to simulate in the test tube a possible effect of water in the mechanism of catalysis on the enzyme surface. The rate of hydrolysis of acetyl phosphate was measured in the presence of 0.1 M NaOH and that of ATP in the presence of 1M HCl. In both cases the hydrolysis was accelerated when the water activity of the medium was decreased by the addition of an organic solvents such as dimethyl sulfoxide or ethylene glycol. The activation energy (Ea) and entropy of activation ($\triangle S$) were calculated from Arrhenius plots of the rates of hydrolysis measured at different temperatures. In the different conditions tested the $\triangle S$ was found to have a high positive value. There was a progressive decrease of both Ea and $\triangle S$ when the water activity was decreased by addition of increasing concentrations of dimethyl sulfoxide. In water the Ea for the hydrolysis of ATP and acetyl phosphate were 16.7 and 18.5 kcal respectively and decreased 6.5 and 3.4 kcal in the presence of 60% v/v dimethyl sulfoxide. The $\triangle S$ decreased from 14 and 44 e.u. to 5 and 2 e.u. after the addition of dimethyl sulfoxide (de Meis and Suzano, 1987).

CONCLUSION

Taken together, the evidence presented raise the possibility that among the different mechanisms available for energy transduction in biological membranes, there is one in which energy is not used directly for the synthesis of high-energy molecules but is used to create and maintain localized media of different compositions. In one medium the bond to be formed would be of low energy and it could therefore be formed spontaneously (low water activity). The bond would then be transferred to a second medium where it

would become of high energy (high water activity). In this mechanism, synthesis can occur in the presence of a large excess of water because it is not dependent on the water concentration in the medium but on the manner in which water is organized around the molecules. Media of different composition can be found in two compartments of the cell as for instance the cytosol and sarcoplasmic reticulum lumen or at the molecular level in the catalytic site of an enzyme. Energy would be used by the enzyme to change its conformation, and thereby to create two different microenvironments at the catalytic site, one before and another after the conformational change (de Meis et al., 1980; de Meis, 1981, 1982, 1986; de Meis and Inesi, 1982).

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NMR STUDIES OF COMPLEXES OF Gd^{3+} WITH NUCLEOTIDES AND SUBSTRATE ANALOGUES ON SARCOPLASMIC RETICULUM Ca^{2+}-ATPase†

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INTRODUCTION

The sarcoplasmic reticulum Ca^{2+}-ATPase[1], which is responsible for active calcium transport across the SR membrane, exists in the SR membrane as a 115,000-dalton protein with associated, essential phospholipid. Calcium transport depends on conformation changes in this protein (de Meis, 1981), but the detailed translocation mechanism is for the most part unresolved. Several authors have demonstrated the existence of a phosphorylated transport intermediate containing occluded calcium ions, i.e. bound Ca^{2+}, which is unable to exchange with Ca^{2+} at either side of the membrane (Inesi et al., 1978; Dupont, 1980; Serpersu et al., 1982), but the nature of the Ca^{2+}-occluded form is largely uncharacterized.

One approach to the details of Ca^{2+} transport is to use a spectroscopically active analogue of Ca^{2+} ion. Our laboratory demonstrated in 1979 that the paramagnetic lanthanide ion, Gd^{3+}, could be used for NMR and EPR studies of the Ca^{2+}-ATPase (Stephens and Grisham, 1979). These studies showed that the ATPase bound two Gd^{3+} ions at high affinity Ca^{2+} sites, and that these calcium sites were in fact occluded sites, with a very low accessibility of solvent water. More recently, we have shown that the soluble, monomeric Ca^{2+}-ATPase also binds Gd^{3+} at occluded sites (Klemens et al., 1986). Vilsen and Andersen (1986) subsequently reported occlusion of Ca^{2+} ions in soluble, monomeric SR Ca^{2+}-ATPase, in corroboration of our NMR results with Gd^{3+}.

We show here that several Gd^{3+}-ATPase complexes, which may represent intermediates of ATP hydrolysis and/or Ca^{2+} transport, can be isolated and characterized using NMR techniques with appropriate substrate analogues.

EXPERIMENTAL PROCEDURES

The Ca^{2+}-ATPase was purified and prepared for spectroscopic studies as described (Klemens et al., 1986). The β,γ-bidentate complexes of Co(III) with ATP and AMP-PCP were synthesized as described (Cornelius et al., 1977). $Co(NH_3)_5PO_4$ was synthesized according to Schmidt and Taube (1963).

Water proton relaxation rate measurements were used to examine the interactions of Gd^{3+} with the ATPase. The longitudinal relaxation rate, $1/T_1$, of the protons of water was measured between the frequencies of 20 and 85 MHz on a variable frequency, pulsed NMR spectrometer of our own design. Gadolinium is a paramagnetic ion and increases the $1/T_1$ rate of water protons in its coordination sphere. In the presence of a macromolecule, the effect of the paramagnetic metal ion on the relaxation rate is enhanced due to an increase in the correlation time of the ion-water interaction. All measurements were obtained at 23 +/- 1°C on samples of 100 microliters. The calculations in this paper and the practical application of NMR theory to structural studies of biomolecules has been reviewed (Mildvan and Engle, 1972).

RESULTS

Formation of a Stable Complex with $Co(NH_3)_4$AMPPCP

In order to form a stable nucleotide complex of the ATPase with Gd^{3+}, the Ca^{2+}-ATPase-Gd^{3+} complex was titrated with $Co(NH_3)_4$AMPPCP (Figure 1A). The sample contained 0.06 mM Ca^{2+}-ATPase and 0.05 mM $GdCl_3$. The enhancement of the longitudinal relaxation rate of water protons decreases to a value of 4.5 at saturating CoAMPPCP. As shown in Figure 1B, in the absence of added CoAMPPCP, there is a small, slow decrease in the observed enhancement of the binary Gd^{3+}-ATPase complex over a period of 200 minutes, after which the observed enhancement is constant. When CoAMPPCP is added, the observed enhancement decreases rapidly to a value which is either constant (at high levels of CoAMPPCP)

or decreases only very slowly for several hours. The rapid decrease in the observed enhancement on addition of CoAMPPCP followed by a relatively constant value of ε^* is

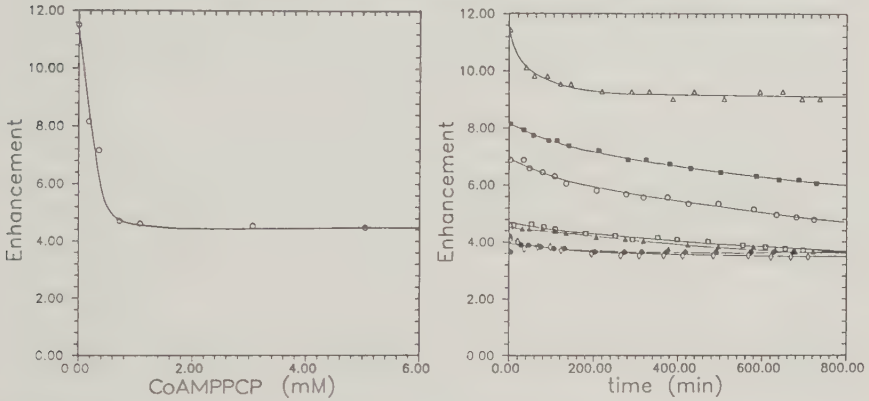

Figure 1: Effect of CoAMPPCP on water proton relaxation by ATPase-bound Gd^{3+}. Initial values are shown in A (left) and time course in B (right). Concentrations of CoAMPPCP: 0.00 mM (△), 0.18 mM (■), 0.36 mM (○), 0.72 mM (□), 1.08 mM (▲), 3.06 mM (◇), 5.04 mM (●).

consistent with the formation of a stable ATPase-Gd^{3+}-CoAMPPCP complex with a water relaxation enhancement factor of approximately 3.6.

Sequential Formation of Multiple Complexes With ATP and $Co(NH_3)_4ATP$

In contrast to the results obtained with the nonhydrolyzable CoAMPPCP, titrations of the ATPase-Gd^{3+} binary complex with either ATP or $Co(NH_3)_4ATP$ resulted in a series of time-dependent changes in the observed enhancement. Figure 2 shows the behavior observed in titrations with ATP. A rapid, concentration-dependent decrease in the observed enhancement (Fig. 2B) is followed by a slower increase and then a decrease in ε^*. The same pattern is observed at all ATP concentrations employed (0.025 mM to 0.658 mM). As the ATP concentration is increased, the enhancement measured immediately following the addition of ATP decreases, and the maximum enhancement measured during the slow phase is decreased. The values of ε^* following the rapid initial decrease on ATP addition are plotted vs. ATP concentration in Figure 2A. The limiting value of ε^* at high ATP is 3.6, similar to that obtained at

high levels of CoAMPPCP in Figure 1A, consistent with the formation of similar complexes in these two cases. The final enhancement of approximately 1 could either represent enzyme-bound Gd^{3+} with a low enhancement factor or Gd^{3+} which has been dissociated from the enzyme. Methods to distinguish these possibilities are described below.

Similar behavior, with a somewhat slower time course, is also observed for $Co(NH_3)_4ATP$. As shown in Figure 3B, a rapid, concentration-dependent decrease in the observed

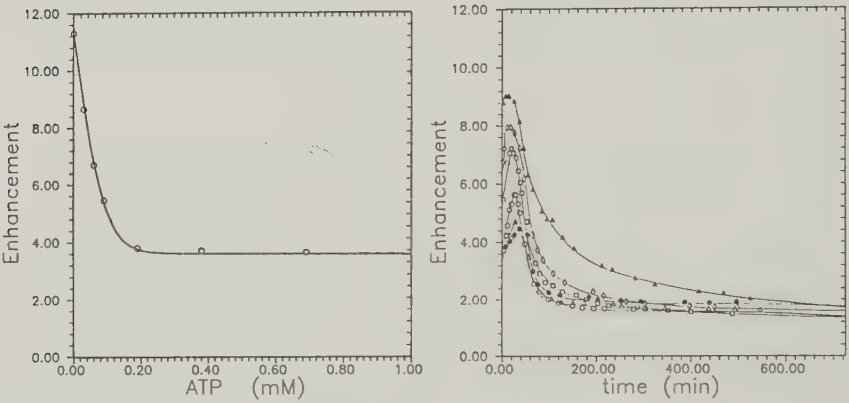

Figure 2: Effect of ATP on enhancement of water relaxation by ATPase-Gd^{3+}. Initial values in A (left) and time course in B (right). Levels of ATP: 0.025 mM (▲), 0.060 mM (◇), 0.094 mM (■), 0.188 mM (○), 0.376 mM (△), 0.658 mM (●).

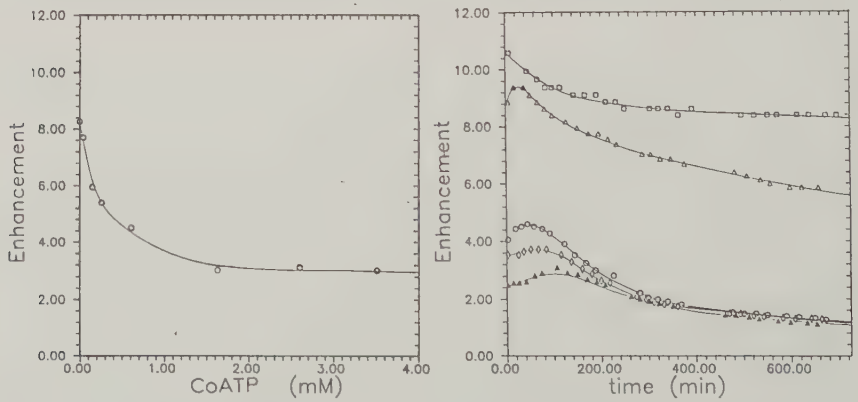

Figure 3: Effect of CoATP on enhancement of water relaxation by ATPase-Gd^{3+}. Initial values in A (left) and time course in B (right). Concentrations of CoATP: 0.00 mM (□), 0.04 mM (△), 0.77 mM (○), 1.53 mM (◇), 3.06 mM (▲).

enhancement is followed by a slower increase and then a decrease in ε^*. The limiting value of ε^* at high levels of CoATP is somewhat lower than that observed for CoAMPPCP andATP (2.5 compared to 3.6). The overall time course, on the other hand, is very similar to that seen with ATP. The slow increase, then decrease, in ε^* on ATP addition was analyzed in terms of the following mechanism:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \qquad (1)$$

Values for k_1 and k_2 were determined by fitting the data of Figures 2B and 3B to a sequential mechanism as in Eqn. 1. At a concentration of CoATP of 0.776 mM, for example, the best fit for k_1 is 0.02 min^{-1} and the best fit for k_2 is 0.0065 min^{-1}.
The point of maximum enhancement in the slow phase occurs at longer times as the concentration of CoATP is increased.

Characterizing Intermediates with Occluded Calcium Sites

We have previously shown that the nuclear relaxation properties of water can be used to detect and characterize occluded sites for Gd^{3+} (i.e., Ca^{2+}) on the Ca^{2+}-ATPase (Stephens and Grisham, 1979; Klemens et al., 1986). The relaxation rates of water protons coordinated to Gd^{3+}, which is in turn bound to Ca^{2+} sites on the ATPase, are dependent on τ_c, the correlation time for the Gd^{3+}-nucleus dipolar interaction. For enzyme complexes, τ_c itself is dominated by τ_s, the spin-lattice relaxation time for the electrons of Gd^{3+}. τ_s is in turn dependent on the rate of transient distortions of the Gd^{3+} coordination geometry due to collisions with solvent molecules. If the Gd^{3+} site is occluded, transient distortions due to water encounters will be infrequent and the experimental τ_c will be large. τ_c is normally determined by a study of the frequency dependence of the metal-induced relaxation rate, $1/fT_{1p}$ (Mildvan and Cohn, 1970). The results of such a study for the complexes studied in this paper are shown in Table I. As described elsewhere (Mildvan and Cohn, 1970) the frequency dependence of $1/fT_{1p}$ may be used to determine the correlation time, τ_c, and the coordination number, q, for water protons on the ATPase-bound Gd^{3+}. The τ_c values for the Ca^{2+}-ATPase obtained here (Table I) and in our previous studies (Stephens and Grisham, 1979; Klemens et al., 1986) are unusually long compared to those observed in other Gd^{3+}-protein complexes (Klemens et al., 1986). The complex with the longest value of τ_c (i.e., τ_s) is the Gd^{3+}-ATPase

-$Co(NH_3)_4$ATP complex. The value of τ_c observed in this case (1×10^{-8} sec) is 30-200 times greater than those for typical Gd^{3+}-protein complexes and reflects what is apparently the most highly occluded Gd^{3+} site observed to date.

In the limit of fast exchange, the Solomon-Bloembergen equation which describes the Gd^{3+}-H_2O dipolar interaction (Mildvan and Cohn, 1970) is

$$1/fT_{1p} = 2[H_2O]/([Gd^{3+}]T_{1p}) = (896/r)^6 q[f(\tau_c)] \quad (3)$$

where q is the number of water protons in the inner coordination sphere of Gd^{3+}, r is the Gd^{3+}-water proton distance, and $f(\tau_c)$ is the correlation function, which is given by:

$$f(\tau_c) = 3\tau_c/(1 + \omega_I^2\tau_c^2) + 7\tau_c/(1 + \omega_S^2\tau_c^2) \quad (4)$$

In this equation, ω_I is the nuclear resonance frequency and ω_S is the electron resonance frequency. Having determined values for τ_c, we can use equations 3 and 4 to calculate q,

Table I

Analysis of the Frequency Dependence of T_{1p}

Complex	τ_c ($s \times 10^9$)	q
ATPase-Gd_{site1}	1.91	3.11
ATPase-Gd_{site2}	2.12	1.66
ATPase-Gd_{site1}-$Co(NH_3)_4$AMPPCP	1.62	1.06
ATPase-Gd_{site1}-$Co(NH_3)_5PO_4$	4.89	0.42
ATPase-Gd_{site1}-$Co(NH_3)_4$ATP	9.90	0.25

the values of which are shown in Table I. These values of q are based on an assumed value of 3.1 Å for the Gd^{3+}-H_2O proton distance (Dwek, 1973). Although the absolute values of q are dependent on this distance, the relative magnitudes of q for the various complexes are accurately described by Table I. Thus, the value of q appears to be different in the various Gd^{3+} complexes observed here. Gd^{3+} at site 1 exhibits a q of approximately 3 in the binary Gd^{3+}-ATPase complex, but this value drops to 0.25 and 0.42 in the complexes with $Co(NH_3)_4$ATP and $Co(NH_3)_5PO_4$.

DISCUSSION

Appropriate choices of substrate analogues have

permitted the isolation and observation of several intermediates in the Ca^{2+}-ATPase cycle, and NMR studies with Gd^{3+} complexes have allowed the characterization of these intermediates. A simple mechanism for the Ca^{2+}-ATPase is shown in Figure 4. The complex formed here with $Co(NH_3)_4$AMPPCP likely represents E_1CaATP. The initial drop in ε^* upon addition of either ATP or $Co(NH_3)_4$ATP (Figures 2 and 3) are also manifestations of the formation of this same complex. The increase with time, then decrease, in ε^* observed in Figures 2 and 3 may represent the formation of complexes analogous to E_1CaADP·P_i and E_1Ca-P, respectively. Consistent with this is the observation that the Ca^{2+}-occluded complex formed by the ATPase with $Cr(H_2O)_4$ATP is an analogue of E_1-P (Vilsen and Andersen, 1986). The values of τ_c and q determined in the present study indicate that the sequential formation of these intermediates in the Ca^{2+}-ATPase pathway involve increasingly occluded and desolvated Gd^{3+} (i.e., Ca^{2+}) ions. This is to be expected for a transport system which must move Ca^{2+} ions across the

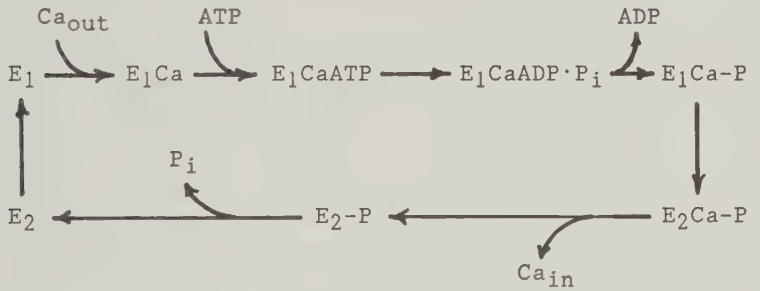

Figure 4: A simple mechanism for the SR Ca^{2+}-ATPase.

sarcoplasmic reticulum membrane. The NMR data presented here permit for the first time a quantitative characterization of the sequential formation of these intermediates.

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[1]Abbreviations: NMe_4^+, tetramethylammonium; AMPPCP, adenyl-5'-yl methylenediphosphate.

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